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SOME ASPECTS OF DEVELOPMENT AND METABOLISM IN NITRATE- AND SULFATE-DEFICIENT *SPIRODELA POLYRHIZA*

by

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Abstract

Senescence, turion dormancy and germination were investigated in nitrate-deficient (1/20 Hoagland's nitrate) and sulfate-deficient (1/200 Hoagland's sulfate) *Spirodela polyrhiza* L. (Schleiden) grown in continuous light at 25-26°C. Availability of substrates, i.e. nitrate or sulfate, or some amino acids normally derived from these anions, was shown to directly affect frond senescence, turion dormancy and turion germination. Cytokinins (benzyladenine or isopentenyladenine at 0.5 or 5.0 mg/l) were ineffective in delaying senescence induced by nutrient deficiency. However, at these concentrations, they prevented turion development, overcame dormancy and induced germination in mature turions formed in nutrient-deficient media. These results indicate that a close relationship exists between nutrient availability (or utilization) and cytokinin activity.

Consequences of nitrate and sulfate deficiency were investigated at the level of amino acid metabolism. While the concentration of protein amino acids declined in both, nitrate- and sulfate-deficient plants, a general decrease in free amino acids was detected only in nitrate-deficient plants. The levels of most free amino acids in sulfate-deficient plants remained comparable to the controls, with the exception of significantly higher concentrations of asparagine and glutamine. The changes in the levels of amino acids in deficient plants were not the result of decreased rates of synthesis, as judged by the *in vivo* incorporation of labeled precursors of the "aspartate pathway", i.e. aspartate, homoserine and cysteine, into product amino acids threonine, isoleucine and methionine. In contrast, expressed on a fresh weight basis, the *in vitro* activity of homoserine dehydrogenase (E.C.1.1.1.3, L-homoserine: $NAD(P)^+$ oxidoreductase) declined in the deficient cultures to about 10% of the control, but apparently not below the rates needed to maintain *in vivo* amino acid synthesis in the deficient plants.

Although exogenously applied 1mM asp, glu and gly were capable of supporting the growth of nitrate-deficient plants and 0.1mM cysteine and methionine supported the growth of sulfate-deficient plants, the controls responded to excess amino acids by enhanced turion development (a characteristic stress response). Methionine, threonine and lysine (1mM) inhibited the growth of control plants and turion development of deficient plants. Labeling experiments with radioactive sulfate in the presence of cold 1mM

threonine plus lysine demonstrated that while the synthesis of methionine via the aspartate pathway is inhibited by these amino acids, there may also be a more direct effect on protein synthesis.

Aminoacylation of tRNA and protein synthesis in control and deficient plants were also investigated. At the whole plant level, tRNA charging decreased slightly by 13% and the rate of protein synthesis decreased by 12%. In contrast, the rate of proteolysis increased in the deficient fronds (7.4 fold in nitrate–deficient cultures). Lower turnover of labeled leucine in the tRNA pool of deficient versus control or recovering plants suggests that tRNA aminoacylation plays an important role in metabolism and development of nutrient–deficient plants.

It is concluded that the withdrawal of macro–nutrients, or conversely, the application of exogenous amino acids, represent methods which effectively disrupt cellular metabolism at a level closely linked to macromolecular synthesis. These methods may serve as a useful tool in elucidating regulatory/metabolic mechanisms underlying plant development.

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List of Abbreviations

BA	N^6–benzyladenine
BSA	bovine serum albumin
DPM	disintegrations per minute
EDTA	ethylene diamine tetraacetic acid
xg	gravitational force
IPA	isopentenyladenine or 6(gamma,gamma–dimethylallyl –amino)–purine
Kd	rate constant of protein degradation
Ks	rate constant of protein synthesis
$NADP^+$	nicotinamide adenine dinucleotide phosphate
PAGE	polyacrylamide gel electrophoresis
PEG 6000	polyethylene glycol, mol.wt. 6000
PVP	polyvinyl pyrrolidone
RuBP	ribulose bis–phosphate
SAM	S–adenosyl methionine
SDS	sodium dodecyl sulfate
S.E.M.	standard error of the mean
Sp	specific activity of protein
St	specific activity of tRNA
TCA	trichloroacetic acid
Tris	tris (hydroxymethyl) aminomethane
tRNA	transfer ribonucleic acid
Vs	rate of synthesis (protein)

1. General Introduction

Studies of macro-nutrient deficiency in plants have been limited in number in the last three decades, perhaps as result of the finding that deficiency can be readily avoided by fertilizer applications. In contrast, nutrient limitation has been employed as one of several possible treatments which disrupt normal bacterial growth, and as a result can be used to investigate the regulation of bacterial metabolism and development (Gallant, 1979; Szulmajster, 1979; Cozzone, 1981). This approach has been rarely considered by plant scientists, perhaps because plant developmental phenomena are perceived to be more complex and regulated by environmental factors such as light and temperature interacting with plant growth regulators. Nevertheless, nutrient availability and/or nutrient utilization are basic requirements of plant growth and development and are known to play a role in apical dominance, flowering, leaf senescence and dormancy (Wareing and Phillips, 1970). The latter two developmental processes, particularly foliar senescence, will be the subject of this investigation.

It is clear from Thimann's recently published book on plant senescence (Thimann, 1980) that our understanding of the metabolic regulation of this process is minimal. Leaf senescence is characterised by chlorophyll loss, decrease in protein and nucleic acid levels with attendant increases in the activity of hydrolytic enzymes, decreased photosynthetic activity and structural changes in organelles. The involvement of environmental factors such as light and temperature and of plant growth regulators such as auxins, gibberellins, cytokinins and abscisic acid has been established. However the 44 year-old hypothesis of nutrient diversion (Molisch, 1938) has not been extensively tested. Molisch's hypothesis has been given little credence despite the observations that some annuals, if given optimal cultural (including nutrient) conditions, will not senesce and may grow for several years. Plant tissues cultured in optimal nutrient media survive much longer than the parent plant (Leopold, 1980). Nitrogen fertilizer applications are also known to delay senescence and onset of dormancy in perennial plants (Kozlowski, 1964).

In the present work, the possibility will be examined that macronutrient deficiency or inability to utilize nutrients may be one of the primary causes of senescence, i.e. the loss of chlorophyll, other macromolecules and eventual loss of cellular structure. Through an investigation of the metabolic consequences of macronutrient deficiency,

namely nitrate and sulfate, the basis for investigation of other levels of senescence regulation will be suggested. The immediate consequences of decreased primary assimilation into glutamate or cysteine are unknown and one can only speculate about the possible sequence of metabolic events which eventually culminate in deficiency-induced senescence. Presumably, the *de novo* synthesis of amino acids derived from glutamate and cysteine decreases, limiting not only protein synthesis but essentially all aspects of metabolism that are dependent on N and S containing compounds. This sequence cannot proceed at random and must be tightly regulated, since senescence is not a simple random degradative process (Thimann, 1980). The exact regulatory relationships may in fact be difficult to establish. While it is likely that senescence at the cellular and organellar level proceeds in an apparently linear fashion, regulation of metabolic events occurs at several levels simultaneously. A small number of key metabolites or growth regulators (pleiotropic or multi-level regulators) may be responsible.

The regulation of metabolic events associated with nutrient deprivation is much better understood in prokaryotic organisms. Bacteria such as *Escherichia coli, Klebsiella aerogenes, Streptococcus faecalis, S. lactis, Pseudomonas aeruginosa* and particularly the soil bacterium *Arthrobacter crystallopoietes* enter a state of "maintenance metabolism" upon reduction in C or N availability and remain in this condition with only a gradual loss of viability (Dawes, 1976). Bacteria of the genus *Bacillus* respond to reduced C,N, or P nutrient availability by spore formation (Sonnenshein and Campbell, 1976; Szulmajster, 1979). At the metabolic level, the consequences of decreased nitrogen nutrition are best understood in *E. coli*. Starvation of this organism is usually achieved by removing amino acids from prototrophic cells, by depriving auxotrophic strains of required amino acids, by the use of amino acid analogs which interfere with synthesis of corresponding amino acids or by impairing synthesis of certain amino acids by feedback inhibition (Gallant, 1979; Cozzone, 1981). It was discovered that it is not the shortage of the amino acid itself, but rather the decreased aminoacylation of tRNA, which triggers the so called "stringent response". This response is characterised by stimulation of transcription of operons for amino acid synthesis and glycogen synthesis and by inhibition of stable RNA accumulation, glucose respiration, carbohydrate, lipid, phospholipid, cell wall and nucleotide synthesis, transport of glycosides and polyamines.

In the above reactions, the relative levels of charged vs. uncharged tRNA act as metabolic regulators, while the rate of translation is affected directly by decreased substrate availability. The stringent response can be ellicited not only by starvation but also by chemical or genetic interference with the aminoacylation reaction. Decreased rate of protein synthesis is accompanied by an increase in protein degradation, resulting in a low, basal level of protein turnover. Overall, functional proteins of lower molecular weight are synthesized. However, the mechanisms of maintaining translational accuracy in the deficient state are not fully understood.

The regulatory functions of the tRNA molecule have received much attention (Cortese, 1979). For example, uncharged tRNA is the signal for ppGpp synthesis in the idling reaction of protein synthesis. This nucleotide may interact directly with RNA polymerase or interact with DNA of the attenuator regions of his, trp, leu and ile–val operons. The exact mechanisms of transcriptional regulation by uncharged tRNA remain to be clarified. It was shown, that the relative concentrations of tRNA species are subject to regulation, i.e. the abundances of tRNA are geared to the frequency of amino acids required in translation (Cortese, 1979). An extreme example of this "developmental or functional adaptation of tRNA" is the synthesis of fibroin in the posterior silk gland of *Bombyx mori* larvae. Fibroin consists primarily of gly, ala, ser and tyr and the amounts of the respective tRNAs mirror the frequencies of the amino acids (Garel *et al.*, 1970). Similar correlation has been found between tRNA levels and protein composition in plant seedlings (Kedzierski, 1981).

Before further discussion of eukaryotic organisms, it should be established, whether their tRNA charging is decreased during nutrient starvation. For example, the above mentioned silkworm larvae, when deprived of mulberry leaves prior to cocoon spinning, show decreased amino acid content in the silk glands, decreased tRNA charging (lower enzyme activity and aminoacyl–tRNA levels) and decreased RNA synthesis (Chavancy and Fournier, 1979). Histidinol, a competitive inhibitor of the aminoacylation of his–tRNA has been used to decrease the level of his–tRNA in cultured animal cells. This condition led to the so called "pleiotypic response" and specifically decreased rRNA synthesis (Grummt and Grummt, 1976) and increased the rate of protein degradation (Scornik *et al.*, 1980). In contrast, Orlovskaya (1979) reported increased incorporation of

^{3}H-leucine into the tRNA of muscle tissue in fasting rabbits. However, this effect may be related to specific reversal of starvation in muscle by leucine and the associated increase in protein synthesis (Tischler *et al.*, 1982).

Regulation of the cell cycle in eukaryotes may also involve tRNA charging. Starvation of wild-type yeast for any of the essential elements prevents entry into the S-phase and unbudded cells accumulate. Unger and Hartwell (1976) reported that cells of *Saccharomyces cerevisiae* will stop growth in the G-1 phase following starvation for sulfate of the prototroph, starvation for methionine of a met auxotroph or shift to the restrictive condition (temperature) of a conditional met-tRNA synthetase mutant. From the above observations it was concluded that if a signal exists for impending sulfate starvation, it occurs beyond the met-tRNA synthetase reaction. Uncharged (met-)tRNA is a likely molecule to act as such a signal. In yeast and *Neurospora,* starvation for one amino acid leads to de-repression of several other amino acid synthetic pathways. Spurgeon and Matchett (1977) have demonstrated that indoleacrylic acid, an inhibitor which causes accumulation of indoleglycerol, inhibits the histidine biosynthetic pathway via inhibition of his-tRNA synthetase and decreased his-tRNA charging levels. Furthermore, the synthesis of histidine, tryptophan and arginine biosynthetic enzymes was de-repressed under these circumstances. A reciprocal situation was observed with a tryptophan pathway inhibitor. The authors suggested that charging of tRNA is involved in multiple pathway de-repression in this organism.

No reports are available on the status of tRNA charging in nutrient-deficient plants or cultured plant cells. Of some relevance in this regard may be the observation that cells of dormant shoot apical meristems of ash were all arrested in the pre-synthetic G-1 phase (Cottignies, 1979). Evidence from other eukaryotic organisms indicates that metabolic regulation by relative tRNA charging levels may be a general phenomenon also applicable to plants. Aspects of plant metabolism in which co-regulation has been suggested will be reviewed in the following paragraphs.

The regulation of plant metabolic pathways is usually studied at the level of enzyme activation and inhibition by pathway end-products. For example, the pathway leading from aspartate to the synthesis of lysine, threonine, isoleucine and methionine is thought to be regulated by changing levels of its end-products (for detailed discussion,

see Section 3.1). However, significant fluctuations in pathway end-products experienced by micro-organisms are rarely a factor in autotrophic plant growth. In plants, it may be rewarding to search for a higher level of regulation, particularly in respect to inter-pathway regulation and the co-ordination of autotrophic, growth-related metabolism. Rather than an over-supply of a single metabolic product, the availability of substrates plays a basic role in plant metabolism and growth. Several areas have been investigated which suggest a close relationship between two or several metabolic pathways related to primary assimilation, namely: a) primary assimilation of nitrate and sulfate, b) photosynthesis and N or S assimilation, c) protein turnover during nutrient limitation and d) RNA metabolism during carbon starvation. Specific examples are discussed below.

The primary pathways of nitrate and sulfate assimilation in plants have been elucidated in recent years. Nitrate assimilation is catalysed by nitrate and nitrite reductases, glutamine synthetase and glutamate synthase (Miflin and Lea, 1976, 1980; Beevers and Hageman, 1980), while sulfate assimilation occurs via sulfate adenylyltransferase, adenosine 5'-sulfatophosphate sulfotransferase, and cysteine synthase (Giovanelli *et al.*, 1980). The relationship between these two pathways has been investigated in cultured tobacco cells (Reuveny *et al.*, 1980). The rate of development of nitrate reductase, induced by nitrate in the media, was directly proportional to the initial sulfate concentration in the medium. Conversely, the de-repression of sulfate adenylyltransferase by low sulfate was dependent on the availability of nitrate. Such regulation would ensure a balanced supply of reduced N and S for tobacco protein, in which these elements occur in a 25:1 ratio. Suggestions regarding the possible identity of the regulatory molecules were not offered. Amino acids also play a role in the (de-)repression of nitrate reductase in tobacco cells (Filner, 1966), a finding that does not exclude the possibility that amino acid metabolites are the actual regulatory entities. Decreased nitrate reductase activity and elevated tissue nitrate levels were detected in sulfate-deprived maize seedlings (Friedrich and Schrader, 1978).

A close relationship also exists between nitrogen availability and relative rates of carbon dioxide fixation into organic acids or carbohydrates. Increased availability of ammonia favors the photosynthetic production of amino acids at the expense of sucrose

in *Chlorella pyrenoidosa* (Kanazawa *et al.*, 1970), alfalfa leaf discs (Platt *et al.*, 1977) and isolated poppy mesophyll cells (Paul *et al.*, 1978). This effect appears to be modulated by increased activity of pyruvate kinase and phosphoenol pyruvate carboxylase. Similar observations were made in isolated spinach leaf cells, although in this case, total CO_2 fixation was increased by added ammonia (Woo and Canvin, 1980a). Additions of glutamate, nitrite and inhibitors of the glutamine synthetase/glutamate synthase enzymes inhibited this increased assimilation of CO_2. Only incorporation into the neutral sugar fraction was affected. These observations led to the suggestion that not only ammonia, but increased nitrogen assimilation was responsible for the relative increase in amino acid photosynthesis (Woo and Canvin, 1980b). Hydroxylamine, an intermediate of nitrate assimilation, has been reported to inhibit oxygenase activity and stimulate carboxylase activity of ribulosebisphosphate carboxylase/ oxygenase in *Anabaena cylindrica* (Okabe *et al.*, 1979) and may play some role in the photorespiratory nitrogen cycle (Keys *et al.*, 1978). In the reverse deficient condition, i.e. in CO_2 free air, sharply reduced incorporation of ^{15}N into amino acids is observed in barley, wheat, corn and bean leaves (Canvin and Atkins, 1974). Sulfate deficiency had long term detrimental effects on photosynthesis in whole leaves and isolated chloroplasts (Terry, 1976), although these effects may be due to chloroplastic deterioration and therefore provide little insight into the immediate relationship between sulfur assimilation and carbon fixation.

Low nutrient availability has been observed to decrease the rate of protein synthesis and to increase the rate of protein degradation in *Lemna minor* (Trewavas, 1972b; Cooke *et al.*, 1979), although it is difficult to obtain absolute values for the rates of protein turnover (Davies, 1979). The radioactive label applied can be metabolised into non-protein compounds, enter non-synthetic pools and the label released by protein degradation can be re-incorporated into protein. The mechanism for increased protein degradation has been linked to the increased permeability of the tonoplast and increased release of proteolytic enzymes (Cooke *et al.*, 1980). No relationship between tRNA charging levels and the rate of protein turnover has been established, however. At the level of nucleic acids, rRNA precursor processing into mature rRNA is decreased in carbon starved *Spirodela oligorhiza* (Rosner *et al.*, 1977). The transcription of new rRNA precursors re-adjusted only slowly in relation to the rapidly inhibited processing.

It is apparent from the enumeration of metabolic pathways affected by substrate limitation, that little is known about their possible co–regulation. Generally, it is assumed that co–regulation occurs by "cascading mechanisms", i.e. changes in the levels of products of one pathway regulate the activities of one or several other pathways. To date, the role of putative pleiotropic regulators, particularly at the level of enzyme (de–)repression, has not been considered in plants. However, the metabolic responses to nutrient limitation have been studied in a variety of plant species. This thesis, in an attempt to unify the observations within one species, *Spirodela polyrhiza*, investigates the consequences of nutrient deficiency at three levels: a) developmental, b) amino acid biosynthesis and c) protein/aminoacyl–tRNA synthesis.

2. Senescence, Turion Development and Germination.

2.1 Introduction

Investigations of the complete plant dormancy cycle including senescence, dormancy, and germination are still lacking. This may be due to the long developmental periods involved. Furthermore, the developmental and metabolic links between senescence of the parent plant and the resumption of growth or germination of the dormant organ have been rarely considered. Among the numerous environmental factors initiating and promoting senescence, and/or subsequently breaking dormancy, nutrient deficiency is the one factor most directly linked to the metabolism of the organism. In this chapter, the dormancy cycle of aseptically grown *S. polyrhiza* is described. The dormancy cycle of this plant can be completed within one month and its development can be easily manipulated by the nutrient and/or growth regulator content of the growth and germination media.

The turions, which are modified fronds lacking aerenchyma and filled with starch (Hillman, 1961), form on the senescing fronds in response to environmental stresses such as low temperature (Shibazaki and Oda, 1979), to nitrate, potassium, calcium deficiencies (Newton *et al.*, 1978), to sulfate, phosphate, magnesium deficiencies (Malek, unpublished observations), or following abscisic acid treatments (Perry and Byrne, 1969). Conceivably, there are several pathways leading to the macromolecular events that are ultimately responsible for turion development. In the present work, deficiencies of nitrate and sulfate were chosen as turion-inducing treatments, since these anions participate in the formation of key amino acids (Bonner and Varner, 1976; Datko *et al.*, 1978; Rhodes *et al.*, 1977). It is proposed that either amino acid deficiency or decreased involvement of amino acids in macromolecular synthesis leads to turion induction. In this regard, it is significant that amino acid deficiencies result in spore formation in *Bacillus* sp. (Szulmajster, 1979) and in some fungi (Moore-Landecker, 1972).

In the germination phase of the cycle, the metabolic consequences of the deficient state have to be overcome. Turions respond to nutrient application by germinating, providing their dormancy has been broken. Again, amino acid metabolism might be implicated in dormancy breaking and the germination stages of development.

Amino acid interconversions may be among the earliest reactions taking place in germinating seeds (Collins and Wilson, 1975).

Superimposed on the nutrient-regulated development of *Spirodela* are the known effects of the plant growth regulator cytokinin. For example, exogenous cytokinins have been shown to induce turion germination in complete nutrient media (Lacor, 1969). Furthermore, the well documented interaction between the nutrient status and cytokinins in senescence and dormancy (Salama and Wareing, 1979; Horgan and Wareing, 1980) suggests that cytokinins act as a signal of normal nutrient status, i.e. normal vegetative pattern of development. This chapter describes the development of *Spirodela* in response to nutrient deficiency and the application of cytokinins. Subsequent investigations of amino acid and protein metabolism utilize cultures growing under physiological conditions described in this chapter.

2.2 Materials and Methods

2.2.1 Growth conditions

The greater duckweed *Spirodela polyrhiza* L. (Schleiden) Strain O-381 (Shibazaki and Oda, 1979) was grown aseptically in 250 ml wide neck Erlenmeyer flasks containing 40 ml full-strength Hoagland's medium (with FeEDTA as iron source) under continuous white light (mixed fluorescent and incandescent source, 100 to 120 $uE.m^{-2}.s^{-1}$ at 25 to 26°C. Macronutrient deficient media contained 1/20 nitrate (0.25 mM KNO_3 and $Ca(NO_3)_2$) or 1/200 sulfate (0.01 mM $MgSO_4$). The resulting cation deficiency was adjusted to control levels by adding the appropriate chloride salt(s). Experimental cultures were initiated by 3 to 4 frond colonies from stock cultures that had been supplemented with 1% (w/v) sucrose to detect possible contamination by microorganisms. Stock exogenous amino acids (9mM) and cytokinins (4mg/l, Sigma, St. Louis) were added in appropriate volumes (5 or 0.5ml) through 0.22 um Millipore membrane filters to give the desired final concentrations. The cytokinins were dissolved in 1ml of 95%(v/v) ethanol during preparation of the stock solution. Fronds were counted in continuously growing cultures and turion production was determined by periodic harvesting of cultures and counting of turions retained on a fine wire mesh (Malek and Oda, 1980). Total chlorophyll contents

were determined according to Harborne (1973).

2.2.2 Germination tests

On day 28 of culture, dormant turions were aseptically collected from several flasks. To break dormancy, the turions were placed in 200 ml of autoclaved and aerated (overnight) 20% (w/w) PEG 6000(Carbowax, Fisher Scientific, Edmonton) for four days (Malek, 1981). Samples of 20 to 30 turions were then transferred into 10 ml of germination medium (see Results) in 125 ml foam stoppered conical flasks. Germination in the light at 25 to 26°C (as above) was then determined after 4 or 11 days.

2.2.3 Amino acid extraction and analysis

Seven or eleven day old fronds (0.5 to 1.0g) were homogenised in a conical ground glass grinder (Bellco, Vineland, N.J.) in 10 ml of hot 80% (v/v) ethanol. The extract was evaporated to dryness *in vacuo* at 50°C on a Buchler flash evaporator (Buchler Instruments, Fort Lee, N.Y.), the residue, washed with 10 ml of anhydrous diethyl ether, was dissolved in 10 ml of water and applied to a column (8x1 cm) of Dowex 50Wx8 (200–400 mesh) H^+ ion exchange resin. The column was washed with 40 ml of water and the amino acids were eluted with 40 ml of 6N HCl. The acid eluate was dried *in vacuo* and taken up in 4 ml of 0.2N trisodium citrate buffer (pH 2.2). To estimate the amounts of free glutamine and asparagine, aliquots of the extract were hydrolysed in 6N HCl for 2 hours as described below and the data for glutamine and aspargine were corrected for hydrolysis during the usual preparative procedure.

The protein–bound amino acids were recovered from the insoluble cell residue by overnight hydrolysis in 6N HCl under nitrogen at 110°C. The hydrolysate was filtered, dried *in vacuo* and prepared for analysis as above. Appropriately diluted extracts were analysed on the Beckman 121 AutoAnalyser (Anonymous, 1970).

2.2.4 Microbiological assay of methionine

The low levels of free methionine, not readily measured by automated amino acid analysis, were determined using the more sensitive microbiological assay. Free amino acids were extracted as above, except that the dried acid eluate was taken up in 2 ml of

water. The assay was carried out according to the Difco manual (Anonymous, 1953), using Difco media and cultures of actively growing *Pediococcus cerevisiae* Balcke ATCC 8042. Under these culture conditions, the microorganism responded to methionine and methionine sulfone.

2.3 Results

The growth of control, nitrate- and sulfate- deficient plants is shown in Figure 1. The concentrations of nitrate and sulfate were determined in preliminary experiments in order to synchronize the initial growth rates (decreasing by day 7) and the time of turion appearance (day 11). The rates of frond senescence, however, differed in the two deficient conditions. Sulfate-deficient plants contained slightly more chlorophyll (Table 1) and protein (Table 8) than nitrate-deficient plants at the time of turion appearance. The control plants ceased normal growth and small fronds and turions started to appear on day 19. The low growth rate of the deficient plants could be increased by addition of sulfate or nitrate to control levels on days 5,7,9 and 11 (Table 2). These cultures developed chlorophyll but turions, initiated by day 9 or 11, completed their development. Selected exogenous amino acids, applied on day 7 of culture, were also capable of reversing the senescence and turion formation that was induced by nitrate deficiency and sulfate deficiency (Table 3). Glutamate, aspartate and glycine at 1mM increased the growth rate of nitrate-deficient plants to the control level, but were unable to prevent turion development. Methionine and cysteine at 1mM inhibited growth of nitrate-deficient plants but were largely without effect at 0.1 and 0.01mM. In contrast, sulfate-deficient plants responded to methionine (0.1mM) and cysteine (1.0 and 0.1mM), but not to aspartate, glutamate and glycine. Methionine at 1mM inhibited growth and turion formation, while 0.01mM methionine or cysteine had only a slight effect on the sulfate-deficient plants. Non-deficient plants were little affected by the exogenous amino acids. Of these, aspartate, glutamate and glycine enhanced turion formation, while 1mM methionine inhibited growth.

The rate of turion formation is shown in Figure 2. The sulfate-deficient plants had a greater capacity for turion production, however the turions were smaller and dark in color. The development of mature, abscising turions took about 6 days. For subsequent

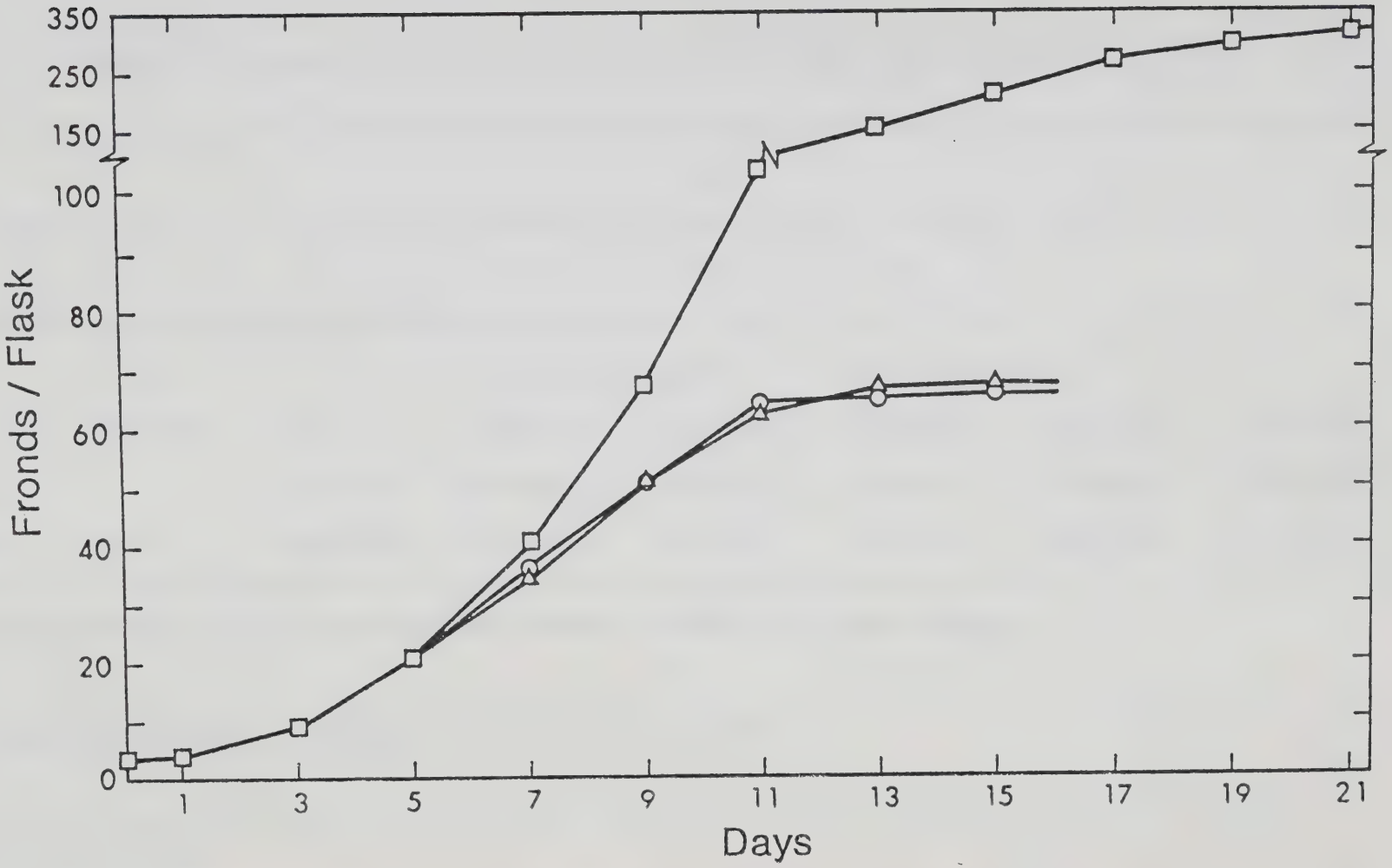


Figure 1. *Growth of control (□), nitrate-deficient (0) and sulfate-deficient (△) Spirodela. Each point represents the average of three experiments, S.E.M. did not exceed ± 3 fronds and for this reason is not depicted on the graph.*

Table 1. *Chlorophyll content of control, nitrate- and sulfate-deficient Spirodela.*

Deficiency	Day						
	0	6	9	12	15	19	23
	mg. g f.wt.$^{-1}$						
None	1.01±0.02	0.98±0.02	0.88±0.05	0.81±0.05	0.84±0.02	0.81±0.02	0.70±0.02
Sulfate	-	0.92±0.03	0.63±0.04	0.48±0.02	0.39±0.02	0.35±0.03	0.31±0.02
Nitrate	-	0.83±0.03	0.49±0.05	0.34±0.01	0.29±0.01	0.26±0.01	0.21±0.01

Chlorophyll was extracted in acetone and determined according to Harborne (1973).

[1]Each number represents the average of three extracts ± S.E.M.

Table 2. *Reversal of nitrate and sulfate deficiency.*

	Deficiency			
	Nitrate		Sulfate	
Time of Addition[1]	Growth rate	Turions formed	Growth rate	Turions formed
day	*fronds/2 days/culture*	*number*	*fronds/2 days/culture*	*number*
5	28[2]	0	28	0
7	30	0	31	0
9	23	4	26	1
11	6	7	12	25

[1]Nitrate-deficient cultures were aseptically given 5 ml of 40 mM $Ca(NO_3)_2$ and KNO_3 each (final 5 mM each) and sulfate-deficient cultures were provided with 5 ml of 16 mM $MgSO_4$ (final 2mM) at times indicated.

[2]Each number is an average of two determinations, the control (non-deficient) growth rate being 37 fronds/2 days culture (from Figure 1) determined between days 9 and 11. Turions were counted on day 15.

Table 3. *Growth and turion formation in the presence of exogenous amino acids.*

Deficiency	Day	Additive(s) (mM)									
		water	glu	asp	gly	met			cys		
			1.0	1.0	1.0	1.0	0.1	0.01	1.0	0.1	0.01
None											
fronds/2 days/flask	9-11	34±4[1]	29±6	35±3	37±5	11±2	36±2	43±1	56±9	40±3	38±1
fronds/flask	15	>120[2]	>120	>120	>120	93±4	>120	>120	>120	>120	>120
turions/flask	15	0	+[3]	+	+	0	0	0	0	0	0
day turions appear		19	15	15	15						
Nitrate											
fronds/2 days/flask	9-11	8±1	29±5	35±3	30±1	4±1	9±1	5±1	0	7±1	5±1
fronds/flask	15	62±6	110±7	129±3	122±4	49±5	56±3	51±4	40±1	55±3	53±1
turions/flask	15	55±4	28±13	12±3	18±7	0	6±2	46±7	0	35±5	41±4
day turions appear		11	11	11	11		13	11		11	11
Sulfate											
fronds/2 days/flask	9-11	12±2	1±1	5±1	5±1	5±1	40±5	17±2	15±4	42±5	16±2
fronds/flask	15	74±7	52±3	59±4	55±1	48±2	>120	96±4	>120	>120	104±6
turions/flask	15	37±5	45±4	50±2	54±4	0	0	13±2	0	0	8±4
day turions appear		11	11	11	11			13			13

Exogenous amino acids were added on day 7 and growth and turion production evaluated as described in Materials and Methods.

[1] Each number represents the average of three flasks ± S.E.M.

[2] Growth in excess of 120 fronds was not evaluated. [3] + Indicates that a few turions (1-3) appeared by day 15.

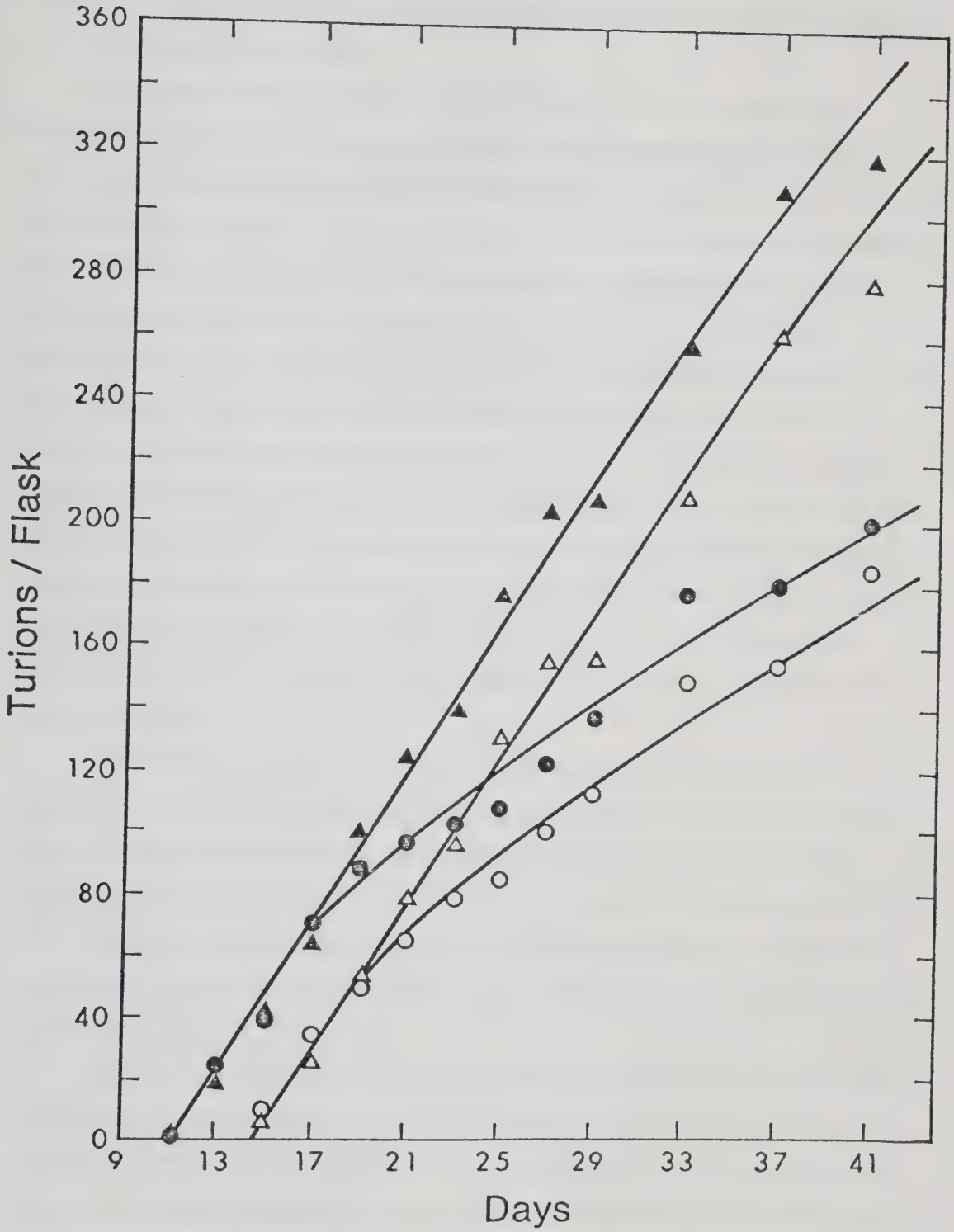


Figure 2. *Turion production by nitrate-deficient* (0) *and sulfate-deficient* (△) *Spirodela. Total visible turions* (●,▲); *total mature, turions, detached from parent plants and sinking to flask bottom* (0,△). *Each point is the value obtained from single culture flask.*

germination studies, turions were collected on day 28 of culture, to avoid ageing in the spent medium and associated changes in germination capacity (Lacor, 1969; Shibasaki and Oda, 1979; Malek and Oda, 1980).

Germination results are shown in Tables 4,5 and 6. In early experiments, dormancy was broken by a four day treatment in sterile, aerated PEG 6000 (Malek, 1981). As shown previously for turions formed in the presence of 1% sucrose, the PEG 6000 treatment resulted in rapid germination in nitrate (turions formed in N-deficiency) and in sulfate (turions formed in S-deficiency) (Table 4). However, turions formed in sulfate-deficient medium also germinated slowly (30% on day 11) in 1mM nitrate. Exogenous salts were not necessary for germination and development, since both types of turions germinated, albeit slowly, in the presence of cytokinins (Tables 4 and 6). Furthermore, the cytokinins overcame the need for the dormancy breaking PEG 6000 treatment (Table 6). The germination of both types of turions in IPA, particularly at 5mg/l, was lower than in BA. When cytokinins were combined with the appropriate, previously lacking nutrient salts, rapid germination ensued. Adding both, sulfate and nitrate together with 0.5mg/l BA led to 100% germination of "nitrate-deficient" turions and 79% germination of "sulfate-deficient" turions on day 32 of culture, i.e. 4 days after turion harvest. (Table 6).

Ability of the products of primary assimilation, i.e. amino acids, to support germination is reported in Table 5. Germination in amino acids was slower than in salt solutions. Turions formed in nitrate deficiency germinated to 31-57% by day 43 in aspartate, glutamate and glycine, but not in sulfur amino acids. The turions formed in sulfate-deficient medium responded only to 1mM methionine (36% germination on day 43). Cysteine at 1mM appeared detrimental to both types of turions, leading to bleaching and loss of viability.

In view of the promoting effect of the cytokinins on turion germination and the documented effects of cytokinins on delaying plant senescence (Thimann, 1980), the response of senescing *Spirodela* fronds to exogenous cytokinins was examined (Table 7). The cytokinins were applied at the time of turion initiation (day 9). The most striking effect was a complete inhibition of turion development. The growth rate of the deficient plants increased slightly, but not significantly in the presence of cytokinins, yet remained

Table 4. *Germination of osmotically pre-treated turions formed in nitrate- or sulfate-deficient media, in nitrate, sulfate and cytokinin solutions.*

Germination Medium	Turions formed in: Nitrate Deficiency		Sulfate Deficiency	
	Days of germination			
	4	11	4	11
	% germination			
1mM $Ca(NO_3)_2$	84±3[1]	-[2]	0	30±g
1mM Mg SO_4	0	0	53±4	-
1mM $Ca(NO_3)_2$ and Mg SO_4	42±7	-	77±5	-
0.5 mg/l BA	0	89±2	0	89±2
5.0 mg/l BA	0	44±4	0	83±6
0.5 mg/l IPA	0	93±1	0	40±2
5.0 mg/l IPA	0	8±3	0	73±2

Turions were harvested on day 28, treated with 20% (w/w) PEG 6000 for 4 days and germinated as described in Materials and Methods for 4 or 11 days. A 4-day pre-treatment in water gave no germination after 11 days.

[1]Each number is an average of four flasks ± S.E.M.

[2]Turions germinating on day 4 were not kept until day 11 of germination.

Table 5. *Germination of osmotically pre-treated turions in amino acids.*

Germination Medium	Turions formed in:	
	Nitrate Deficiency	Sulfate Deficiency
1mM	*% germination (on day 11)*	
Glutamate	57±13[1]	0
Aspartate	40±11	0
Glycine	31±12	0
Methionine	0	36±5
Cysteine	0	0

Turions were harvested on day 28, treated with 20% (w/w) PEG 6000 for 4 days and germinated as described in Materials and Methods for 11 days (no germination was detected on day 4). Only 5 ml of germination medium were used.

[1]As in Table 4.

Table 6. *Germination of untreated turions in nitrate, sulfate and cytokinin.*

Germination Medium	Turions formed in: Nitrate Deficiency		Sulfate Deficiency	
	Days of Germination:			
	4	11	4	11
	% germination			
0.5 mg/l BA	0	92±3[1]	0	78±3
5.0 mg/l BA	0	84±3	0	79±2
0.5 mg/l IPA	0	62±4	0	18±4
5.0 mg/l IPA	0	3±1	0	7±1
0.5 mg/l BA+ 1mM $Ca(NO_3)_2$	98±1	-[2]	n.d.[3]	n.d.
0.5 mg/l IPA+ 1mM $Ca(NO)_2$	80±1	-	n.d.	n.d.
0.5 mg/l 1mM $MgSO_4$	n.d.	n.d.	40±4	-
0.5 mg/l 1mM $MgSO_4$	n.d.	n.d.	73±4	-
0.5 mg/l BA+1mM $Ca(NO_3)_2$+1mM $MgSO_4$	100±0	-	79±2	-
0.5 mg/l IPA+1mM $Ca(NO_3)_2$+1mM $MgSO_4$	99±1	-	71±3	-

Turions were collected, washed and placed directly into germination medium as described in Materials and Methods. Ethanol (1% v/v) used in cytokinin preparation did not induce germination within 11 days.

[1]As in Table 4. [2]As in Table 4. [3]Not determined.

well below that of the controls. The deficient cultures treated with cytokinins had more fronds than control deficient plants, i.e. thin, small fronds were formed instead of turions. The fresh weights of these cytokinin treated, deficient cultures, were not significantly greater than those of untreated deficient plants on day 15. IPA and BA at 0.5 mg/l increased, although not significantly the final fresh weights of control cultures. This increased growth was accompanied by decreased levels of chlorophyll (Table 7). Neither cytokinin at 5.0 mg/l had an effect on the chlorophyll content of the control plants. In the nitrate-deficient cultures, exogenous cytokinins did not maintain higher chlorophyll concentrations. This was in contrast to sulfate-deficient plants, in which cytokinins maintained slightly higher chlorophyll levels (except at 0.5 mg/l). However, the chlorophyll concentrations of cytokinin treated sulfate-deficient cultures never approached the control levels. The few turions that developed in the deficient cultures treated with 0.5 mg/l IPA had germinated in the culture medium by day 15.

Preliminary to biochemical investigations, the amino acid contents of the control, nitrate- and sulfate-deficient plants were determined. Of special interest were changes in the amino acid pools, that might be linked to the development of senescence and dormancy. Although growth rates of control and deficient plants only began to diverge on day 7 of culture, the levels of free and protein-bound amino acids were markedly different by this stage (Table 8 and Table 9). These trends were more pronounced on day 11, the time of turion appearance. Expressed on the basis of individual culture flasks, the nitrate-deficient cultures contained lower amounts of free and protein-bound amino acids than the controls (Table 9). In contrast, the sulfate-deficient cultures contained elevated levels of free amino acids, but the protein-bound amino acid content was as low as that of the nitrate-deficient plants. In total, sulfate-deficient cultures contained 46% of the amino acids of the controls (Table 9). In the controls, amino acid contents doubled between days 7 and 11, sulfate-deficient cultures increased their amino acids by 53% and nitrate-deficient plants by only 2% (Table 9). Expressed on a fresh weight basis, the control plants maintained relatively constant concentrations of free and protein-bound amino acids. With the exception of the free amino acids in sulfate-deficient plants, there was a general decline in the concentration of amino acids in the deficient plants. (Table 8).

Table 7. *Growth and turion formation of control and nutrient-deficient plants exposed to cytokinins on day 9 of culture.*

Treatment	Deficiency	Growth Rate Day 9-11	Turion Formation Day 15	Growth Day 15	Growth Day 15	Total Chlorophyll Day 15
		fronds/ 2 days/ flask	*turions/ flask*	*fronds/ flask*	*mg f.wt./ flasks*	*µg/g f.wt.*
(0.09% ethanol)	Nitrate	12±1[1]	23±2	74±2	487±18	379±35
	Sulfate	12±2	18±3	91±7	651±56	412±16
	None	25±2	0	>120	682±136	865±49
0.5 mg/l IPA	Nitrate	15±2	3±1	96±2	532±31	371±20
	Sulfate	15±3	3±1	103±8	604±44	486±12
	None	27±2	0	>120	1149±35	751±61
5.0 mg/l IPA	Nitrate	15±1	0	100±3	499±16	343±13
	Sulfate	14±1	0	106±6	520±38	466±30
	None	16±1	0	120±3	612±63	940±110
0.5 mg/l BA	Nitrate	16±1	0	108±5	594±9	310±11
	Sulfate	20±4	0	116±8	694±52	443±31
	None	31±2	0	>120	1118±63	774±41
5.0 mg/l BA	Nitrate	12±2	0	103±3	482±10	353±17
	Sulfate	15±3	0	116±5	604±42	486±22
	None	22±2	0	>120	748±42	887±61

Cytokinins were added in 5 ml aliquots on day 9 as described in Materials and Methods.

[1]Each number represents the average of 3 treatments ± S.E.M.

Table 8. *Endogenous amino acid contents of control, nitrate- and sulfate-deficient Spirodela.*

Amino acid	Deficiency: None				Deficiency: Nitrate				Deficiency: Sulfate			
	Day of Culture: 7		11		7		11		7		11	
µmole/flask	free	protein	free	protein	free	protein	free	protein	free	protein	free	protein
lysine	0.09[1]	1.70	0.04	2.09	0.05	0.07	0.03	0.62	0.05	0.70	0.07	1.19
histidine	n.d.[2]	2.16	n.d.	4.82	n.d.	1.38	n.d.	0.68	n.d.	1.40	n.d.	1.45
arginine	0.11	2.13	n.d.	4.10	n.d.	1.25	n.d.	0.53	n.d.	1.06	0.08	1.23
asparagine	0.18	n.d.	0.27	n.d.	0.02	n.d.	0.03	n.d.	0.81	n.d.	4.84	n.d.
aspartate	0.25	2.80	0.66	5.99	0.07	1.32	0.06	1.57	0.44	1.63	0.51	1.95
threonine	0.04	1.82	0.10	3.52	0.03	0.79	0.03	0.91	0.06	0.99	0.07	1.15
serine	0.13	1.88	0.23	4.02	0.09	0.96	0.10	1.13	0.13	1.16	0.36	1.48
glutamate	0.29	3.10	0.79	6.98	0.15	1.50	0.14	1.69	0.37	1.77	0.60	2.09
glutamine	0.18	n.d.	0.37	n.d.	0.04	n.d.	0.05	n.d.	0.28	n.d.	1.97	n.d.
proline	n.d.	1.69	n.d.	3.63	n.d.	0.79	n.d.	0.92	n.d.	1.02	n.d.	1.18
glycine	0.03	3.14	0.07	6.60	0.03	1.52	0.04	1.67	0.06	1.81	0.15	2.17
alanine	0.13	3.01	0.33	6.39	0.05	1.47	0.06	1.66	0.10	1.77	0.55	2.04
cystine	n.d.	0.15	n.d.	0.26	n.d.	0.04	n.d.	0.02	n.d.	0.03	n.d.	0.03
valine	0.03	1.90	0.07	4.39	0.07	0.98	0.04	1.16	0.04	1.48	0.07	1.36
methionine	0.0028[3]	0.32	0.0078	0.61	0.0032	0.14	0.0033	0.06	0.0035	0.15	0.0051	0.04
isoleucine	0.01	1.29	0.030	2.69	0.03	0.63	0.03	0.71	0.02	0.73	0.03	0.93
leucine	0.01	2.31	0.01	4.64	0.01	1.11	0.01	1.28	0.02	1.29	0.02	1.72
tyrosine	n.d.	0.44	n.d.	0.93	n.d.	0.19	n.d.	0.22	n.d.	0.28	n.d.	0.30
phenylalaine	n.d.	0.07	n.d.	0.19	n.d.	0.04	n.d.	0.06	n.d.	0.07	n.d.	0.08
Total (µmoles/flask)	1.47	31.17	2.96	61.86	0.64	14.84	0.61	15.17	2.34	17.09	9.30	20.39
Fresh weight (g/flask)	0.313		0.645		0.293		0.371		0.265		0.414	
Total (µmoles/g f.wt.)	4.70	99.58	4.59	95.91	2.18	50.65	1.64	40.09	8.83	64.48	22.46	49.25

Amino acids and amides were extracted and analysed as described in Materials and Methods.

[1] Each number represents the average of three extracts. S.E.M. did not exceed 10%.

[2] Not detectable.

[3] Free methionine was determined by microbiological assay as described in Materials and Methods (average of four extracts, maximum S.E.M. recorded was 16%).

Table 9. *Percentage change in total (free and protein-bound) amino acids per flask with time and in comparison with the control (data from Table 8).*

Amino acid	Deficiency: None	Nitrate		Sulfate	
	% change (day 7 to 11)	*% change (day 7 to 11)*	*% of control (on day 11)*	*% change (day 7 to 11)*	*% of control (on day 11)*
lys	119	80	31	168	26
his	223	50	14	104	30
arg	183	42	13	124	32
asn	150	150	11	598	1793
asp	218	117	25	119	37
thr	195	115	26	116	34
ser	211	117	29	143	43
glu	229	111	24	126	35
gln	206	125	14	704	532
pro	215	117	25	116	33
gly	210	110	26	124	35
ala	214	113	26	139	39
cys	173	50	8	100	12
val	231	114	27	94	32
met	191	43	10	27	7
ile	227	112	25	128	33
leu	200	115	28	133	37
tyr	211	116	24	107	32
phe	271	150	31	114	42
Average change	199	102	24	153	46

The changes in total amino acids shown in Table 9 mainly reflect changes in protein concentration and composition, since free amino acids represented only a small fraction of these totals. An exception was the increase in free glutamine and asparagine in the sulfate-deficient plants, which accounted for a large portion of the amino acid content. Also notable was the decline in protein cysteine and methionine in the deficient plants. Other detectable protein-bound amino acids remained in relatively constant proportions in the control and deficient plants. In the free pools, the major, metabolically important amino acids such as asp, glu, gly, ser and ala varied most dramatically. Free methionine concentration, however, remained relatively constant.

2.4 Discussion

Autotrophic growth of *Spirodela* in nitrate- and sulfate-deficient media clearly resulted in senescence and turion development. The time of turion appearance in both types of deficient culture was synchronized. However, the rates of senescence of the parent fronds differed, indicating that turion initiation depends on specific cellular components rather than overall protein and chlorophyll degradation (Table 1). Earliest turions were initiated on about day 9 of culture, and the development of such "committed" turions could not be reversed by subsequent addition of the lacking anion (Table 2). During such recovery, frond primordia not committed to turion formation initiated new fronds and the parent fronds re-greened. When applied prior to turion initiation (day 7), certain amino acids were also capable of partially reversing the nitrate deficiency. For example, the growth rates of nitrate-deficient plants returned to the control rate in the presence of 1 mM glutamate, aspartate and glycine but turion formation was not prevented, only decreased (Table 3). This could be due to an insufficient concentration of these amino acids. However, the response of control plants to these exogenous amino acids (accelerated turion appearance) and of sulfate-deficient plants (further depressed growth and increased turion formation) may indicate that imbalance in amino acid or protein metabolism enhances turion initiation. Free exogenous amino acids may not be metabolized rapidly enough as a nitrogen source and sufficient free amino acid may remain to support developmental pathways which maintain turion formation.

Sulfate deficiency was reversed by 1.0 and 0.1mM cysteine and by 0.1mM methionine. Methionine at 1mM inhibited growth and turion development in all cultures. This response may be attributed to the inhibition of enzymes of the "aspartate pathway" and will be investigated in the following chapter. The lowest concentration (0.01mM) of the sulfur amino acids examined was insufficient to elicit a significant response. It appears that amino acids at 1 or 0.1 mM concentrations can support growth of nitrate- and sulfate-deficient *Spirodela*, although the metabolic and developmental consequences of unbalanced (in relation to protein metabolism) amino acid supply remain to be clarified. Variable growth responses in a related species *Lemna minor* to nutrient salts and amino acids was reported by Joy (1969). The work of Fukunaga and King (1982) with cultured plant cells indicates that exogenous amino acid effects are not likely mediated by only one enzyme, namely the inhibition of nitrate reductase, but have a more general effect on plant cell metabolism.

Utilization of nutrients in senescing tissues appears to be promoted by cytokinins (Galston and Davies, 1970; Naito *et al.*, 1979; Skoog and Schmitz, 1979; Kao, 1980; Thimann, 1980). It was of interest to determine if senescence could be delayed by cytokinins in nutrient-deficient plants, which presumably lack the substrates necessary for the reversal of senescence. Judging by the low chlorophyll levels, the progress of senescence, induced by nitrate deficiency, was apparently not arrested by the exogenous cytokinins. In contrast, the sulfate-deficient plants treated with cytokinins had more chlorophyll than the untreated plants, although senescence was not prevented. These plants had greater pools of free amino acids, some of which may act as amino-donors in chlorophyll synthesis. The control cultures treated with 0.5 mg/l cytokinins, which stimulated growth, contained significantly less chlorophyll than the untreated controls. In this case, the rapid growth rate may have decreased the availability of nitrogenous compounds for chlorophyll synthesis. The most striking effect of cytokinins on the deficient plants was the complete reversal of turion initiation and development. This effect could be achieved by both cytokinins tested, BA and IPA at 5 or 0.5 mg/l, applied as late as day 9 of culture (Table 7). Thin, anthocyanin rich fronds developed instead, suggesting that cytokinins may be inducing qualitative rather than quantitative changes in genome expression. Cytokinins may arrest the developmental pathway leading to turion

formation by acting as a signal associated with normal nutrient status. The endogenous levels of cytokinins are known to decrease in nitrate-deficient plants (Salama and Wareing, 1979; Horgan and Wareing, 1980), yet it is not clear, if decreased nutrient utilization is responsible for decreased cytokinin synthesis or *vice versa*.

As a prerequsite to studies of amino acid and protein metabolism, the levels of free and protein bound amino acids were investigated in the control and deficient plants. The patterns of change of the free and protein amino acids were not surprizing (Tables 8 and 9) in view of the early observations of Crane and Steward (1962) and Steward (1963). A dramatic increase in free organic nitrogen in sulfate-deficient tomatoes was noted by Nightingale *et al.* (1932) and later in other species by Eaton (1951) and by Adams and Sheard (1966). The overall decline in free amino acids of nitrate-deficient plants is also well established (Ergle, 1953). Assimilation of nitrate and sulfate into amino acids characteristically declines as deficiency progresses (Bonner and Varner, 1976; Datko *et al.* 1978; Rhodes *et al.*, 1977). However, a direct relationship between decreased amino acid availability and decreased macromolecular levels has been difficult to demonstrate. The decreasing protein concentration of deficient plants (Steward, 1963), detached leaves or senescing plant organs (Woolhouse, 1978) suggests that balanced protein turnover becomes disrupted. In deficient *Spirodela* fronds, protein concentration declined between days 7 and 11 (on per g f. wt. basis),yet, the total protein content of deficient cultures (on per flask basis) increased slightly (Table 8). The amino acids were apparently re-distributed into new fronds. Free amino acids in the medium were measured to contribute only 30 to 50 nmoles of the total per flask, i.e. leakage of amino acids into the medium was not a factor in this re-distribution.

Any re-distribution of amino acids into new fronds or turions requires net loss of protein in the parent fronds. The question than arises, which proteins in the older fronds are sacrificed? In the cellular senescence process, the quality and numbers of chloroplasts and free ribosomes are observed to decline first, followed by mitochondria, plasmalemma and the nucleus (Butler and Simon, 1970). The degradation of chloroplastic protein, particularly RuBP carboxylase/oxygenase, is a major contributor to the increase in free amino acids in senescing tissue (Peterson and Huffaker, 1975; Morita, 1980). Therefore, chloroplastic proteins may be the ones sacrificed during young frond

development under nutrient stress. Also related to protein turnover may be the increasing levels of glutamine and asparagine, which were detected not only in the sulfate-deficient plants, but also in the nitrate-deficient plants between days 7 and 11 (Table 9). The striking increases in free glutamine and asparagine have also been linked to sufficient activities of enzymes involved in secondary amide synthesis (Thomas, 1978). The most interesting change, however, was the decrease in protein cysteine and methionine of both types of deficient cultures. This loss might be attributed to loss of a specific, sulfur-rich protein. Such protein may have importance in maintenance of function of the chloroplast and possibly the re-establishment of autotrophic growth during turion germination. It is possible that putative sulfur-rich protein(s) may be more severely affected in the later stages of nutrient deficiency and senescence. In contrast, Hanson *et al.*(1941) have observed that the N/S ratio of chloroplastic protein decreased rapidly during early senescence of Sudan grass. A specific lupin seed storage protein rich in sulfur (gamma conglutin), is known to specifically decrease when the seeds develop in sulfate-deficiency (Gilespie *et al.*, 1978; Randall *et al.*, 1979). It may be appropriate to mention at the conclusion of the discussion of nutrient deficiency induced senescence, that frond senescence and turion development may be unique in their direct relationship. In other higher plants, the relationship between nutrient status, leaf senescence and dormant organ development are generally more complex due to translocation phenomena (Sesay and Shibles, 1980) or monocarpic senescence (Nooden *et al.*, 1978).

The consequences of nutrient deficiency during turion development have to be overcome during germination, yet, simple addition of the previously limiting anion did not support germination. Rapid loss of this type turion dormancy was achieved by a four day treatment with 20% PEG 6000 (Malek, 1981). It is possible that osmotic shock modifies amino acid metabolism in such a manner as to allow germination (Hanower and Brzozowska, 1975). Aeration of the sterile, de-gassed PEG solution was found to be necessary for the osmotic treatment to be effective. The role of gas regimes and osmotic shock in the breaking of turion dormancy remains to be resolved.

The nutrient deficiency experienced during turion development still had some effect during germination, since the addition of nitrate or glutamate, aspartate and glycine at 1mM concentration allowed germination of pre-treated turions formed in

nitrate-deficient media and conversely, sulfate and methionine supported germination of turions from sulfate-deficient cultures (Tables 4 and 5). Cysteine (1mM) bleached and killed both types of turions, possibly by interference with oxido-reduction reactions. Free anions and amino acids may have supported germination simply by initiating *de novo* synthetic reactions. Nitrate, however, also supported low germination of turions formed in sulfate-deficient medium. Stimulatory effects of nitrate on electron transport, similar to those suggested by work on germinating seeds may be responsible (Hendrix and Taylorson, 1974; Esashi *et al.*, 1979). However, exogenous salts were not needed to support germination of turions (Tables 4 and 6). BA and IPA stimulated slow but high final germination, and the turions were presumably utilizing stored endogenous nutrients. Furthermore, the cytokinin treatment had overcome the need for a dormancy breaking treatment. The proposal can be made, that the dormancy breaking treatments (cold or PEG 6000) allowed endogenous cytokinin production and attendant nutrient utilization.

Cytokinins at 5 mg/l, possibly at supraoptimal levels, were less effective than at 0.5 mg/l in stimulating germination. The most rapid and complete germination was achieved when the cytokinin and appropriate salt were provided simultaneously (Table 6). This compares favorably with the observations made by Lacor (1969). The present data, however, stress the relationship between the cytokinin action and specific nutrient utilization by the dormant turions. Exogenous cytokinins were also found effective in the breaking of dormancy of birch trees subjected to nitrate deficiency (Horgan and Wareing, 1980), in allowing the re-growth of nutrient deficient grass tiller buds (Sharif and Dale, 1980) and are occasionally reported to affect seed germination (Khan, 1968). It is possible that cytokinin action is linked to the utilization of nutrients (amino acids) at the level of aminoacylation of tRNA, in view of the presence of cytokinin moieties next to the anti-codon of some tRNA molecules (Weil, 1979). Observations reported here can be used in further investigations of the action of cytokinins on tRNA metabolism during senescence and germination.

3. Activity of the Aspartate Pathway in Deficient Duckweed

3.1 Introduction

The so called aspartate pathway for synthesis of lysine, threonine, isoleucine and methionine has been well characterised in plants (Fig. 3). Interest in this metabolic sequence is due to the relative deficiency of these amino acids in many staple seeds which are of dietary importance to man and other monogastric animals. The pathway is also of interest due to the complexity of its regulatory mechanisms as elucidated by studies of microorganisms. (Umbarger, 1978). The end-product, allosteric inhibition of enzymes catalysing the early or branch point steps of the pathway, the presence of isoenzymes sensitive to the various end-products and the enzyme repression mechanisms present in bacteria and lower eukaryotes gave impetus to much of the research on this pathway in plants (Miflin *et al.*, 1979; Bryan, 1980).

To summarise, lysine and threonine, individually or in combination (usually at 1 to 10 mM) inhibit the *in vitro* activity of aspartate kinase, the first enzyme of the pathway (Enzyme number 2 in Figure 3). In *Lemna minor*, lysine at 0.3mM inhibited the enzyme by 37% but threonine at 0.5mM was without effect. In combination, these two amino acids inhibited aspartokinase by 63% (Wong and Dennis, 1973). The presence of two aspartokinases, each sensitive to a different effector was demonstrated in carrot roots (Davies and Miflin, 1978). Multiple enzyme forms have been also reported for the second most widely studied enzyme of the pathway, homoserine dehydrogenase. The low molecular weight form (designated I) was found to be insensitive to threonine inhibition. However, the enzyme lacking form I, from older tissues of maize, was also not sensitive to threonine (DiCamelli and Bryan, 1975).

The *in vitro* activity of homoserine dehydrogenase can be also inhibited by aspartate, serine and cysteine (Bryan, 1980). The physiological significance of this is not clear. Of special interest is the activation of threonine synthetase by S-adenosyl methionine (SAM) and its inhibition by cysteine (Madison and Thompson, 1978). These authors suggested that if cysteine were present (or SAM lacking) the decreased activity of threonine synthetase would make more phosphohomoserine available for methionine synthesis; once adequate levels of methionine and SAM were present, more threonine

could be formed. Presumably, the increased threonine levels then inhibit aspartokinase and homoserine dehydrogenase, shutting off the entire pathway. It is however unlikely that concentration changes of this magnitude occur in plant tissues. However, the rate of product utilization could be crucial in the regulation of this pathway. In this case, consumption of amino acids in macromolecular synthesis may be expected to have a regulatory role.

The possibility of a higher level of metabolic controls operating in autotrophic plants has been discussed by Miflin (1977). Nutrient availability has the most direct effect on plant growth and metabolism, i.e. higher nutrient levels are known to positively affect assimilatory enzymes. Light, temperature and ionic conditions provide additional levels of regulation. However, the positive effect of available nutrients (or conversely, negative effects of nutrient defficiency) on the overall rate of amino acid synthesis and utilization in macromolecular synthesis, have so far been rarely considered at the molecular level. Aspartate derived amino acids should not be an exception to regulation at this level. Consequently, the following were investigated: 1) the *in vivo* activity of the aspartate pathway using radiolabeled precursors, and 2) the effects of exogenous end-product amino acids on this pathway and on protein synthesis.

3.2 Materials and Methods

3.2.1 Plant material and growth in exogenous amino acids

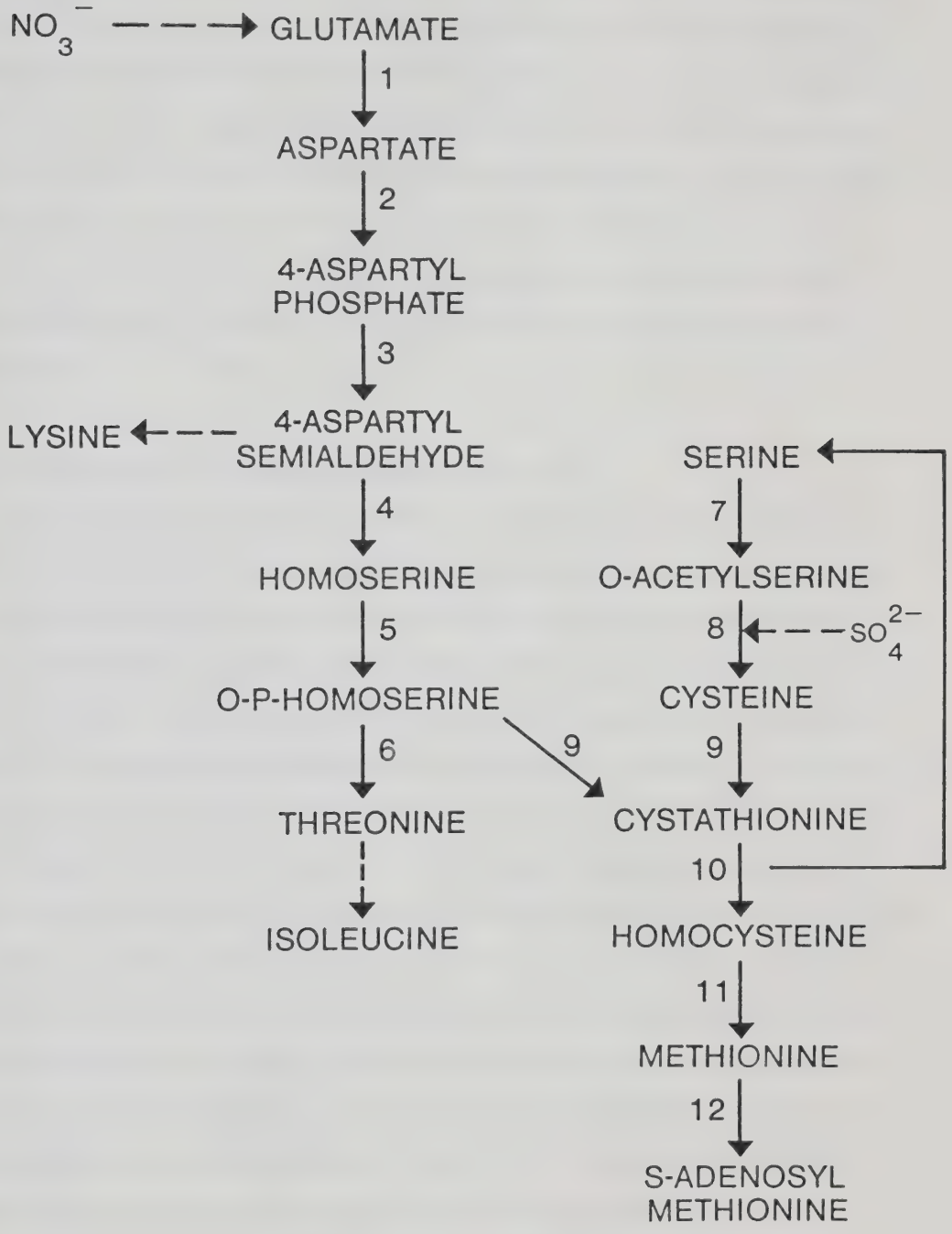
Spirodela polyrhiza was grown as described in Section 2.2.1.

3.2.2 Labeling experiments

Carrier free L-[U-^{14}C] homoserine (40 mCi/mmole, 1uCi), L-[U-^{14}C] aspartic acid (229 mCi/mmole, 5uCi), or sodium [^{35}S] sulfate (45 mCi/mmol, 16uCi, purchased from Amersham, Illinois) were supplied in 10 ml of 11 day old medium (with added amino acids where applicable) to about 0.6 g of 11 day-old *Spirodela polyrhiza* plants. The length of incubation with these substrates is given in the appropriate Tables. Excess radioactivity was removed by washing 3x with 30 to 50 ml of water. Amino acids were extracted and analysed as described in Section 2.2.3. The Beckman AutoAnalyser was used without the

Figure 3. *The pathway of synthesis of aspartate-derived amino acids.*

Reaction No.	Trivial enzyme names	Systematic Enzyme names	E.C. Number
1.	Aspartate transaminase	Glutamate:oxaloacetate aminotransferase	2.6.1.1.
2.	Aspartate kinase	ATP:L-aspartate 4-phosphotransferase	2.7.2.4
3.	Aspartate-semialdehyde dehydrogenase	L-aspartate-β-semialdehyde:$NADP^+$ oxidoreductase (phosphorylating)	1.2.1.11
4.	Homoserine dehydrogenase	L-homoserine:NAD^+ oxidoreductase	1.1.1.3
5.	Homoserine kinase	ATP:L-homoserine O-phosphotransferase	2.7.1.39
6.	Threonine synthase	O-phosphohomoserine phospho-lyase (adding water)	4.2.99.2
7.	Serine transacetylase	Acetyl-CoA:L-serine O-acetyl transferase	2.3.1.30
8.	Cysteine synthase	O-acetyl-L-serine acetate lyase (adding hydrogen-sulfide)	4.2.99.8
9.	Cystathionine γ-synthetase	[O-succinyl-L-homoserine succinate-lyase (adding cysteine)]	4.2.99.9
10.	Cystathionine β-lyase	Cystathionine L-homocysteine-lyase (deaminating)	4.4.1.8
11.	Tetrahydropteroyl-triglutamate methyl-transferase	5-Methyltetrahydropteroyl-tri-L-glutamate: L-homocysteine S-methyltransferase	2.1.1.14
12.	Methionine adenosyl-transferase	ATP:L-methionine S-adenosyltransferase	2.5.1.6


NO_3^-
GLUTAMATE
1
ASPARTATE
2
4-ASPARTYL PHOSPHATE
3
LYSINE
4-ASPARTYL SEMIALDEHYDE
4
HOMOSERINE
5
O-P-HOMOSERINE
6
THREONINE
ISOLEUCINE
9
SERINE
7
O-ACETYLSERINE
8
SO_4^{2-}
CYSTEINE
9
CYSTATHIONINE
10
HOMOCYSTEINE
11
METHIONINE
12
S-ADENOSYL METHIONINE

ninhydrin reaction. Two ml fractions were collected using an LKB Fraction Collector (Bronna, Sweden) and .1 ml aliquots were dissolved in .10 ml of Bray's solution (i.e. 4 g of 2,5-diphenyloxazol, 0.2 g 1,4-di [2-(5-phenyloxazolyl)] benzene, 60 g naphtalene, 100 ml methanol, 20 ml ethylene glycol and dimethoxyethane to make .1 l). Radioactivity was determined in a Tracor Analytic Mark III Scintillation Spectrophotometer. Radioactive peaks were identified by co-chromatography with authentic amino acids in the analyser system or after de-salting (on H^+ 50Wx8, 200-400 mesh Dowex resin, using 40 ml water wash followed by 20 ml of 2N ammonium hydroxide) by cellulose (MN 300, Macherey, Nagel & Co., W. Germany) thin layer chromatography using 80% phenol as solvent system.

3.2.3 Homoserine dehydrogenase extraction and assay

The method of Mathews *et al* (1975) was employed with minor modification. Eleven day old control (8 g) or deficient (.16 g) plants were homogenized by three, 30 second bursts in a Waring Blendor (at circa 0°C in 30 or 60 ml (respectively) of 0.2 M Tris HCl buffer, pH 8.5, containing .10%(w/v) PVP, 20%(v/v) glycerol, 0.15 M KCl, 0.5 mM EDTA and 0.7 mM 2-mercaptoethanol. Cell debris was removed by centrifugation at 20,000x g for 30 minutes and the supernatant brought to 55% saturation with ammonium sulfate (with constant stirring, near 0°C). The precipitate was collected after 30 minutes by centrifuging the extract at 20,000x g for 20 minutes. Precipitated protein was dissolved in 2 ml of the above buffer (lacking PVP and glycerol) and passed through .1x8 cm column of Sephadex G-.15 (Sigma, St. Louis). One ml fractions were collected. This procedure effectively separated salts and anthocyanins from the protein, which was located in fractions 2-6. All steps were carried out at circa 0°C. Protein concentration was estimated by the method of Lowry *et al.* (1951), with BSA Fraction V (Sigma, St. Louis) as a standard. The reaction mixture (.1 ml) for the reverse assay (homoserine oxidation) contained: 200 umoles TrisHCl pH8.5, .150 umoles KCl, 0.7 umoles 2-mercaptoethanol, 0.5 umoles EDTA, 0.6 umoles $NADP^+$, 20 umoles L-homoserine and *circa* 450 ug or 600 ug of protein from deficient or control plants, respectively. Assays were run at 25°C and changes in absorbance at 340 nm were monitored on a Pye Unicam Recording Spectrophotometer (Cambridge, U.K.) over .10 minutes. The reaction rate was

found to be the same in the forward direction at pH 7, using NADPH and D,L-aspartyl semialdehyde, prepared by ozonolysis of allylglycine HCl according to Black and Wright (1955). Neutralization of the semialdehyde removed a strong oxidizing agent, possibly residual ozone, which oxidized NADPH non-enzymatically. Reaction rate was dependent on enzyme concentration. Protein from deficient plants did not appear to contain enzyme inhibitors, since mixing protein from control and deficient plants gave the expected levels of enzyme activity.

3.2.4 Incorporation of tritiated leucine into TCA insoluble protein.

Ten uCi of carrier free L-[4,5-^{3}H] leucine (Amersham, Illinois, 170 Ci/mmole) were added to 2 g of 10 day-old tissue in 10 ml of sterile medium of the same age. After 15, 30 or 60 minutes, excess radioactivity was removed by washing the plants 3x with 30 to 50 ml of water. Tissue was homogenized in a Ten Broeck grinder (Corning, Ontario) at circa 0°C in 15 ml of 0.1M TrisHCl buffer (pH 8.5) and centrifuged at 20,000g for 10 minutes. An aliquot of 0.1 ml was withdrawn for estimation of total leucine uptake and 5 ml of 40%(w/v) TCA were added to the remaining supernatant. The precipitate was collected after 30 minutes by centrifugation at 5,000x g for 10 minutes, resuspended in 5%(w/v) TCA and collected again. The pellet was dissolved overnight in 10 ml of 2N NaOH and 0.1 ml aliquot was counted as described in Section 3.2.2. Protein concentration was determined according to Lowry *et al.*, 1951, using BSA fraction V (Sigma, St. Louis), as a standard. The fraction of label associated with insoluble cell debris was determined by extraction of the 20,000x g pellet 2x with 10 ml of hot ethanol (80%,v/v), collecting the cooled insoluble material by centrifugation at 20,000x g for 10 minutes and redissolving the pellet overnight in 10 ml of 2N NaOH. Insoluble wall material was removed by centrifugation at 20,000x g for 10 minutes and protein and radioactivity were determined as above. These procedures are essentially those employed by Kemp and Sutton (1971).

3.3 Results

The large differences in the free amino acid contents of control, nitrate-deficient and sulfate-deficient cultures (Table 8) were also evident when the data were expressed on a fresh weight basis (Table 10). In the control fronds, the free aspartic acid concentration increased slightly between days 7 and 11. Sulfate-deficient plants had greater levels of aspartate than the controls, while nitrate-deficient plants contained significantly less aspartate (Table 10). The concentrations of amino acids derived from aspartate varied less dramatically in the deficient plants. For example, the levels of free methionine and lysine remained relatively constant and comparable to the controls. Free isoleucine increased two-fold in the deficient plants, while threonine concentration decreased in nitrate deficiency and increased in sulfate deficiency (Table 10).

Carbon flow in the aspartate pathway was examined by supplying ^{14}C-homoserine (Table 11). Deficient cultures absorbed and metabolised 2-4 times as much labeled homoserine as the controls, depending on the feeding period. At any one time, the radioactivity in the free amino acid fraction remained at a comparable level (47 to 69% of total) in all three types of culture. The proportion of homoserine-derived amino acids entering protein was less in the deficient plants, and greater amounts of the label were recovered in non-amino compounds. Analysis of individual amino acids (Table 12) showed that ^{14}C-homoserine was rapidly incorporated into threonine. The amount of labeled homoserine not metabolized remained greater in the deficient plants than in the control. However, the amount of label in homoserine declined rapidly between 30 and 60 minutes in both deficient cultures. Small amounts of label appeared in methionine and isoleucine. Free methionine labeling in the sulfate-deficient plants and controls decreased between 30 and 60 minutes, although this change was not significant in the sulfate-deficient plants. Radioactive threonine, isoleucine and methionine accumulated in the protein of the control plants but at a lower rate in the protein of deficient plants.

The data for homoserine feeding implies that steps in the aspartate pathway linking this substrate to isoleucine, threonine and methionine are not significantly impaired by nitrate or sulfate deficiencies. However, the two main regulatory steps – aspartate kinase and homoserine dehydrogenase are essentially by-passed when homoserine is supplied (Miflin *et al.*, 1979; Bryan, 1980). Feeding labeled aspartate overcomes this

Table 10. *Levels of amino acids metabolically related to aspartate.*

Amino acid	Fraction	Deficiency					
		None		Nitrate		Sulfate	
		Day 7	Day 11	Day 7	Day 11	Day 7	Day 11
		μmoles.g f.wt.$^{-1}$					
asp	free	0.81 (17)[1]	1.02 (22)	0.24 (11)	0.17 (10)	1.65 (19)	1.22 (5)
	protein	8.96 (9)	9.29 (10)	4.52 (9)	4.24 (11)	6.15 (10)	4.72 (10)
lys	free	0.28 (6)	0.06 (1)	0.17 (8)	0.09 (6)	0.17 (2)	0.16 (1)
	protein	5.42 (6)	3.24 (3)	2.59 (5)	1.67 (4)	2.65 (4)	2.88 (6)
thr	free	0.14 (3)	0.15 (3)	0.10 (5)	0.07 (4)	0.23 (3)	0.18 (1)
	protein	5.81 (6)	5.45 (6)	2.70 (5)	2.45 (6)	3.75 (6)	2.77 (6)
ile	free	0.03 (1)	0.04 (1)	0.10 (5)	0.08 (5)	0.07 (1)	0.08 (0)
	protein	4.12 (4)	4.17 (4)	2.15 (4)	1.90 (5)	2.76 (4)	2.24 (5)
met	free[2]	0.009	0.012	0.011	0.009	0.013	0.012
	protein	1.02 (1)	0.94 (1)	0.48 (1)	0.16 (0)	0.57 (1)	0.10 (0)
Total amino acids		104.28	100.50	52.83	41.73	73.31	71.71

Amino acids were extracted and analyzed as described in Materials and Methods. Each number represents the average of three extracts. The S.E.M. did not exceed 10%.

[1] Numbers in brackets indicate % of total free and % of total protein amino acids, respectively.

[2] From microbiological assay. S.E.M. did not exceed 16%.

Table 11. *Distribution of ^{14}C-homoserine label in control, nitrate- and sulfate-deficient plants.*

Deficiency	Labeling period	Radioactivity[1] Organic acids and sugars	Free amino acids	Protein amino acids	Total
	minutes	*(DPM x g f.wt.$^{-1}$) x 10^{-3}*			
None					
	10	171±11 (34)[2]	283± 2 (57)	41± 2 (8)	495
	30	403±84 (33)	572± 32 (47)	236±45 (20)	1211
	60	314±55 (20)	687± 89 (45)	543±48 (35)	1544
Nitrate					
	10	667±41 (32)	1380± 9 (66)	33± 2 (2)	2080
	30	993±11 (39)	1377± 50 (54)	164±30 (7)	2534
	60	1284±43 (42)	1394± 81 (48)	315±24 (10)	2993
Sulfate					
	10	608±58 (29)	1433±116 (69)	43± 1 (2)	2084
	30	826±65 (35)	1416±131 (58)	159±12 (7)	2436
	60	1110±39 (37)	1494±158 (50)	393±35 (13)	2997

[1] 1 µCi of ^{14}C-homoserine was given to about 0.6g of 11 day-old plants in 10 ml of medium. Each number is an average of three extracts ± S.E.M.

[2] Percent of total.

Table 12. *Distribution of ^{14}C-homoserine label in the aspartate-derived amino acids.*

Deficiency	Labeling period	thr	hse	met	ile
		Free amino acids			
	Minutes	*(DPM x g f.wt.$^{-1}$) x 10^{-3}*			
None					
	10	153± 3[1]	33± 3	13± 1	4± 1
	30	421±29	43± 6	28± 5	5± 1
	60	557±59	40± 3	10± 1	10± 1
Nitrate					
	10	125± 2	431±13	18± 1	7± 1
	30	424±35	431±58	35±18	7± 3
	60	690±88	257±12	37± 8	8± 2
Sulfate					
	10	238±15	349±11	24±11	7± 1
	30	631±27	248±27	85±37	9± 4
	60	998±80	161±14	59±20	12± 4
		Protein amino acids			
None					
	30	151±13	8± 3[2]	53±18	17± 7
	60	399±28	2± 1	85±11	79±24
Nitrate					
	30	79± 4	9± 4	32±15	2± 1
	60	243±23	6± 1	48±10	4± 2
Sulfate					
	30	99±11	8± 3	38± 4	3± 2
	60	280±36	8± 1	83± 7	13± 4

[1] Each number is an average of three extracts ± S.E.M.

[2] Two washes of the protein pellet were insufficient to remove all free homoserine.

difficulty. Unfortunately, aspartate readily enters intermediary metabolism in *Spirodela* and a variety of products become labeled (Table 13). It should also be noted that aspartate was capable of reversing the nitrate deficiency (Table 7) and for this reason only the results obtained with control and sulfate-deficient plants are presented and discussed. Despite differences in specific activity and amounts of ^{14}C supplied, ^{14}C-aspartate was taken up less readily than homoserine (Table 13). A significant proportion of the aspartate label was recovered in glutamate and other amino acids not directly related to the aspartate pathway. The fraction of total label appearing in threonine, isoleucine and methionine was small and did not differ significantly in the free pools or protein of control versus sulfate-deficient plants (Table 13). The possibility that methionine synthesis was impaired in the nitrate-deficient cultures was examined using ^{35}S-cysteine. Label was applied for short periods, to avoid saturation of the endogenous pools and to facilitate better evaluation of the initial rate of methionine synthesis (Table 14). The fraction of cysteine sulfur (% of total) entering free methionine was similar in both, control and nitrate-deficient plants although uptake of the label was greater in the latter.

The labeling experiments indicated comparable activity of the aspartate pathway in the deficient plants and the controls. To evaluate wheather *in vitro* enzyme activities correspond to this apparent situation *in vivo*, homoserine dehydrogenase was extracted and assayed. Specific activities were found to be lower in the deficient plants than in the control (Table 15). Taking into consideration the low protein content of the deficient plants (about 40% of control), their capacity to synthesize homoserine on a fresh weight basis appeared to be an order of magnitude lower than that of the controls.

Exogenous, aspartate-derived amino acids are well known to inhibit growth of non-deficient plants. However, exogenous threonine or lysine may be expected to support growth of nitrate-deficient plants. This was not the case, since these amino acids (at 1mM) inhibited growth of both, control and deficient *Spirodela* (Table 16). Inhibition was sufficient to completely arrest turion development of nitrate-deficient plants and to partially inhibit and delay turion formation of sulfate-deficient plants. In contrast, .1mM threonine and homoserine accelerated turion development in control cultures. This response was similar to that obtained with other amino acids (Table 6). At

Table 13. *Distribution of ^{14}C-aspartic acid label in control and sulfate-deficient plants.*

Amino acid	Non-deficient		
	Free amino acids	Protein amino acids	Sugars and organic acids
	DPM x g f.wt.$^{-1}$		
asp	112,300±13,000	10,100±800	318,600±25,900
glu	38,800± 7,100	2,800±500	
thr	7,100± 2,300	2,900±300	
met	1,360± 530	500± 90	
ile	1,440± 420	780±620	
Total	147,000± 8,400	30,500±2,200	
	Sulfate-deficient		
asp	164,400±13,800	19,800±2,900	327,900±22,300
glu	83,400±10,900	9,200±1,900	
thr	11,100± 1,800	4,000±1,300	
met	320± 150	1,030± 390	
ile	1,390± 100	3,000± 530	
Total	279,100±17,200	49,300±11,000	

5 µCi were supplied to about 0.6 g of 11 day-old plants in 10 ml of medium for 1 hr. Each number is an average of four extracts ± S.E.M.

Table 14. *Metabolism of ^{35}S-cysteine by 11 day-old control and nitrate-deficient plants into soluble products.*

	Deficiency					
	None			Nitrate		
Labeling period (minutes)	5	10	15	5	10	15
	(DPM x g f.wt.$^{-1}$) x 10^{-3}					
Total[1]	1760[2]	3060	3888	3890	7702	12683
Cysteine	270 (15)[3]	466 (15)	528 (14)	336 (9)	1337 (17)	2043 (16)
Cystine	148 (8)	191 (6)	139 (4)	0 (0)	744 (10)	1367 (11)
Methionine	71 (4)	103 (6)	537 (14)	232 (6)	420 (6)	1164 (9)
Glutathione	0 (0)	37 (1)	227 (6)	13 (0)	991 (13)	978 (8)

[1] Ten µCi were fed to 0.6 g of tissue in 10 ml of media, for details see Materials and Methods.

[2] Values from single extracts. Large fraction of label represents anionic species derived from cysteine (which may include sulfate or cysteic acid?).

[3] Numbers in brackets represent % of total.

Table 15. *Homoserine dehydrogenase activity extracted from control and deficient plants.*

	Deficiency	
None	Nitrate	Sulfate
	$(\Delta OD_{340}/min/mg\ protein)_x\ 10^{-3}$	
4.3±0.6 [1]	1.9±0.2	1.1±0.3

Assay based on that of Matthews et al. 1975.

In 1 ml reaction of mixture:
- 200 µmoles Tris HCl, pH 8.5
- 150 µmoles KCl
- 0.7 µmoles 2-mercaptoethanol
- 0.5 µmoles EDTA
- 0.6 µmoles $NADP^+$
- 20 µmoles homoserine
- about 600 µg protein from control plants (from 8 g f. wt.)
- about 450 µg protein from deficient plants. (16 g f. wt.)

[1] Each value is an average of duplicate determinations from three different extracts.

Table 16. *Growth and turion production of control, nitrate- and sulfate-deficient plants in the presence of arginine[1] and amino acids derived from the aspartate pathway.*

Deficiency	Day	Additive(s) (mM)														
		water	met		hse		thr		lys		thr lys	thr lys met	thr lys met	thr lys met arg	thr lys met arg	arg
			1.0	0.1	1.0	0.1	1.0	0.1	1.0	0.1	1.0 1.0	1.0 1.0 1.0	1.0 1.0	1.0 1.0 0.1 0.1	1.0 1.0 0.1 0.5	0.5
None																
fronds/2 days	9-11	34±4	11±2	36±2	8±1	30±5	18±3	41±5	12±1	40±2	6±1	10±1	18±2	15±3	16±1	33±3
fronds	15	>120[2]	93±4	>120	79±7	>120	120±8	>120	111±4	>120	59±6	87±5	>120*	>120*	>120*	>120*
turions	15	0	0	0	3±1	0	3±1	0	0	0	0	0	0	0	0	0
day turions appear		19			15		15									
Nitrate																
fronds/2 days	9-11	8±1	4±1	9±1	1±1	11±1	1±1	12±3	4±1	4±2	3±1	6±1	4±1	9±2	4±1	18±2
fronds	15	62±6	49±5	56±3	45±3	66±3	42±2	70±1	50±2	67±3	43±2	54±3	64±3	80±5	78±2	116±2
turions	15	55±4	0	6±2	0	36±9	0	33±2	0	9±3	0	0	0	0	0	10±1
day turions appear		11		13		11		13		13						13
Sulfate																
fronds/2 days	9-11	12±2	5±1	40±5	6±1	10±3	10±1	12±2	9±2	8±3	4±1	7±1	5±1	6±1	8±1	8±1
fronds	15	74±7	48±2	>120	47±3	61±3	57±3	71±8	59±3	65±6	48±3	64±2	87±4	98±5	107±6	58±2
turions	15	37±5	0	0	25±5	44±4	7±2	30±3	2±1	18±3	0	0	0	0	0	36±3
day turions appear		11			11	11	13	11	13	13						11

Amino acids were added on day 7 as described in Materials and Methods. Each number represents the average of three replicates ± S.E.M.

[1] Arginine was tested in view of its activity in cultured *Arabidopsis* cells.

[2] As in Table 6.

* indicates that miniature fronds were developing, i.e. growth was not strictly comparable to the control.

0.1mM, aspartate-derived amino acids had no, or only slight effect on frond growth and turion development, with the exception of methionine, which at this concentration restored vegetative growth of sulfate-deficient plants. Combinations of .1mM threonine and lysine (or together with .1mM methionine) inhibited growth of control cultures more effectively than each amino acid alone. Turion formation was prevented under these conditions. These combinations of amino acids also prevented turion formation and lowered further the already low growth rate of the deficient plants (Table 16). Methionine at 0.1 mM concentration partially relieved the lysine plus threonine inhibition of growth in the control cultures. The initial growth rate remained depressed (days 9-11), however, large numbers of small, curled fronds developed by day 15. This combination of amino acids was not effective in increasing the growth rate of deficient plants to control rates and prevented turion development. Arginine, which has been reported to act additively with methionine in reversing threonine plus lysine inhibition of *Arabidopsis* callus growth (Cattoir-Reynaerts *et al.*, 1981) was not effective in *Spirodela* (Table 16). Applied alone, 0.5 mM arginine had no effect on control and sulfate-deficient cultures, but partially reversed the nitrate-deficient condition as judged by frond production.

The following discussion pertains to Tables 17, 18 and 19 . These tables contain data dealing with two aspects of sulfate metabolism: a) the effects of threonine plus lysine on methionine synthesis and b) the consequence of nitrate- or sulfate-deficiency in sulfate assimilation. In respect to the latter, nitrate-deficient plants may be expected to have reduced requirement for sulfate assimilation, in order to maintain balanced cellular N/S ratio. The limited requirement for sulfate was reflected in the decreased uptake of labeled sulfate by nitrate-deficient plants, presumably due to cellular pools already saturated with cold sulfate (Table 17). Reduced incorporation of ^{35}S into amino acids of nitrate-deficient plants (Table 18) was due to lowered uptake of the precursor sulfate. In contrast, sulfate-depleted plants absorbed large quantities of labeled sulfate and appeared to synthesize cysteine and methionine at rates characteristic of recovering, rather than sulfate-deficient plants.

In reference to point a) above, exogenous threonine plus lysine are thought to inhibit methionine biosynthesis via allosteric inhibition of aspartokinase, the first enzyme of the aspartate pathway (Henke and Wilson, 1974; Mills and Wilson, 1978; Bright,

Table 17. *Uptake and assimilation of ^{35}S-sulfate by control, nitrate- and sulfate-deficient plants, in the absence or presence of 1 mM threonine plus lysine.*

Fraction	Deficiency					
	None		Nitrate		Sulfate	
	no addition	plus 1 mM thr and lys	no addition	plus 1 mM thr and lys	no addition	plus 1 mM thr and lys
	(DPM x g f.wt.$^{-1}$) x 10^{-3}					
Uptake	242±59	181±14	174±44	201±38	26,887±974	20,801±1,413
		(75)[1]		(115)		(77)
Free amino acids	22± 3	14± 1	16± 2	11± 1	4,698±298	2,572± 168
		(64)		(69)		(55)
Protein amino acids[2]	25± 8	9± 3	8± 2	3± 1	1,060± 73	422± 69
		(36)		(38)		(40)

16 µCi of sodium ^{35}S-sulfate were fed for 1 hour to about 0.6 g of tissue in 10 ml of 11 day-old media. Threonine and lysine were given 14 hours prior to extraction and were present in the incubation medium. Each number represents the average of at least three extracts ± S.E.M. Counts were adjusted to time 0.

[1] Percent of incorporation in plants not treated with threonine plus lysine.

[2] Counted prior to Dowex H^+ separation of amino acids.

Table 18. *Incorporation of ^{35}S-sulfate in free amino compounds of control and deficient plants, in the absence or presence of 1 mM threonine plus lysine.*

Amino acid	Deficiency					
	None		Nitrate		Sulfate	
	no addition	plus 1 mM thr and lys	no addition	plus 1 mM thr and lys	no addition	plus 1 mM thr and lys
	DPM x g f.wt.$^{-1}$					
Methionine	2,332±200	1,686±245 (72)[1]	1,741±167	925±162 (53)	433±35x10^3	122±13x10^3 (28)
Cysteine	15,205±3,305	19,204±4,074 (126)	13,745±2,325	10,215±1,382 (74)	2,033±150x10^3	1,104±222x10^3 (54)
Glutathione[2]	3,260±279	2,324±558 (71)	1,705±485	1,765±334 (104)	1,435±291x10^3	788±68x10^3 (55)

[1] Percent incorporation in plants not treated with threonine plus lysine.

[2] Identified on the basis of elution position and co-chromatography using Beckman Auto-analyser.

Table 19. *Incorporation of ^{35}S-sulfate label in protein amino acids of sulfate-deficient plants, in the absence or presence of 1 mM threonine plus lysine.*

Amino acid	no addition	plus 1 mM thr and lys
	DPM x g f.wt.$^{-1}$	
Cysteine	47,810±1,443	16,044±3,823 (33)[1]
Cystine	175,570±7,140	91,517±16,027 (52)
Methionine	127,656±14,382	16,644±3,960 (13)

[1] Percent of incorporation in plants not treated with threonine plus lysine.

Shewry and Miflin, 1978). In keeping with this hypothesis, ^{35}S-sulfate incorporation into free and protein methionine was reduced in control and deficient plants (Table 18 and 19). The overall decrease in ^{35}S-sulfate incorporation into protein of control and deficient plants in the presence of threonine plus lysine (Table 17) may have resulted from decreased methionine availability, or may have been due to a direct effect of threonine and lysine on protein synthesis. The latter possibility has not been considered in published reports. To investigate this point, incorporation of ^{3}H-leucine into soluble protein was studied in the presence of these amino acids at 1mM concentration and also in 0.1 mM methionine, which partially reversed growth inhibition by threonine plus lysine (Table 16). Threonine plus lysine inhibited incorporation into soluble protein very rapidly in the deficient plants and somewhat slower in the controls (Table 20). Although uptake of the labeled precursor was also inhibited, the inhibition of incorporation exceeded the inhibition of uptake in all cases. Methionine did not overcome the inhibition of protein synthesis. Surprisingly, after 72 hours of exposure, when the plants treated with threonine, lysine and methionine were already recovering (Table 16), their apparent protein synthesis was more inhibited than that of the rapidly deteriorating lysine plus threonine treated plants (Table 20).

3.4 Discussion

The concentrations of aspartate-derived amino acids lysine, threonine, isoleucine and methionine remained at relatively constant levels during nitrate and sulfate deficiency. This observation may suggest the presence of a regulatory mechanism in the deficient plants which prevents wide fluctuations in these amino acids. In view of the presumed decreased availability of glutamate and cysteine during nitrate or sulfate deficiencies, respectively, a decreased capacity for the *de novo* synthesis of all other amino acids may be expected. In regard to aspartate-derived amino acids, the feeding experiments provided little evidence to support this contention. Radioactive aspartate, homoserine and cysteine were all incorporated into the other amino acids, irrespective of the nutrient status of the cultures. Although amino acids may be generated by protein degradation during senescence, it is likely that biosynthetic pathways remain active to assure the synthesis of balanced amounts of amino acids needed in *de novo* protein synthesis. The

Table 20. *Inhibition of [^{3}H]-leucine uptake and incorporation into TCA insoluble fraction by threonine, lysine and methionine in control and deficient plants.*

Culture Supplement	Period of exposure to supplement	Deficiency		
		None	Nitrate	Sulfate
	hrs		*% inhibition*	
1 mM thr + 1 mM lys	1 to 2	19 (13)[1]	68 (24)	71 (25)
	14	81 (47)	94 (74)	97 (69)
	72	14 (0)		
1 mM thr + 1 mM lys + 0.1 mM met	1 to 2	28 (19)	59 (31)	54 (20)
	14	85 (64)	96 (81)	96 (78)
	72	55 (22)		

[1]Ten Ci of [^{3}H]-leucine were given for 30 minutes to about 2 g of 10 day-old tissue in 10 ml of appropriate medium supplemented with the amino acids. Each number is derived from at least two sets of data. The first number represents % inhibition of specific activity in TCA precipitate, the number in brackets is % inhibition of label taken up (per g fresh weight). Absolute DPM values for untreated culture are: control 35,000 DPM/mg protein and 2,346,000 DPM/g f.wt.; nitrate-deficient 220,000 DPM/mg protein and 1,100,000 DPM/g f.wt.; sulfate deficient 145,000 DPM/mg protein, 725,000 DPM/g f.wt.

apparent decrease in homoserine dehydrogenase activity *in vitro* may not be sufficient to limit flow through the pathway *in vivo*. The *in vitro* activity of homoserine dehydrogenase can be calculated to be 9.8, 1.6 and 1.0 nmoles/g f.wt./minute for control, nitrate- and sulfate-deficient plants, respectively, while the *in vivo* rates of homoserine conversion to threonine determined during the first 10 minutes of feeding were only about 0.17, 0.14 and 0.27 nmoles/g f.wt./minute for control, nitrate- and sulfate-deficient plants, respectively. This suggests that indeed there is sufficient enzymatic activity present in the deficient plants to fulfill the requirements of the plant. Alternatively, the low *in vitro* activity of the enzyme from deficient plants may indicate that partial denaturation took place during extraction in the presence of secondary metabolites present in increasing amounts during senescence. Perhaps the only pathways whose activity declines due to substrate limitation are the primary assimilation pathways in the chloroplast. In *Spirodela*, only uptake, but not assimilation of ^{35}S-sulfate into cysteine and methionine, was inhibited by nitrate-deficiency. Under these circumstances, the synthesis of sulfate assimilatory enzymes may be limited (repressed by high sulfate and prevented by lowered amino acid availability), yet the enzyme activity may be at a sufficient level to maintain metabolic flow through the pathway (Reuveny *et al.*, 1980). Sakano (1979, 1981)detected changes in aspartokinase isoenzyme activity *in vitro* during the growth of cultured carrot discs and of *Vinca rosea* suspension cells. Low levels of lysine in the cells correlated with increased lysine-sensitive aspartokinase activity. This was interpreted as evidence of de-repression. It would be of interest to determine if the cultures with de-repressed enzyme levels have a greater *in vivo* capacity for amino acid synthesis.

Exogenous aspartate-derived amino acids were inhibitory to growth of control *Spirodela* cultures at 1mM but not at 0.1mM concentration. Also the response to combinations of these amino acids was in keeping with observations made with other species (Dunham and Bryan, 1971; Wong and Dennis, 1973; Henke and Wilson, 1974; Green and Phillips, 1974; Bright, Wood and Miflin, 1978). Methionine at 0.1mM reversed the threonine plus lysine growth inhibition of the control plants, but this was only partial and after a lag period. Even though 0.1mM methionine alone was able to serve as sulfur source for the sulfate-deficient plants, the presence of added threonine and lysine

inhibited growth and turion development. Conversely, nitrate-deficient plants were unable to use threonine and lysine as a nitrogen source, even in the presence of 0.1 mM methionine. These observations suggest a more direct effect of threonine and lysine on metabolism and growth than the usually evoked secondary response to decreased methionine biosynthesis due to allosteric inhibition (Henke and Wilson, 1974; Mills and Wilson, 1978; Bright, Shewry and Miflin, 1978).

As expected, metabolism and incorporation into protein of labeled sulfate was negatively affected by exogenous threonine plus lysine. Both, methionine synthesis and protein synthesis as measured by ^{35}S-sulfate incorporation were inhibited by 2mM threonine plus lysine in wheat plants (Bright, Shewry and Miflin, 1978) and 0.5 or 0.1mM threonine plus lysine inhibited incorporation of aspartate, leucine and tyrosine, but not methionine, into protein of isolated pea chloroplasts (Mills and Wilson, 1978). The above data, while apparently supporting the interpretation that decreased methionine synthesis leads to the limitation of protein synthesis and growth, do not exclude the possibility of a more direct role of excess threonine and lysine in protein synthesis.

As shown in Table 20, exogenous methionine was not able to relieve the apparent inhibition of protein synthesis by threonine plus lysine. After 72 hour exposure to threonine plus lysine, inhibition of protein synthesis was almost overcome, yet the plants were deteriorating. In contrast, the plants provided with methionine in addition to threonine and lysine had their protein synthesis inhibited by 55%, while recovering. From this paradox, it appears that exogenous aspartate-derived amino acids may regulate not only the quantity but also quality of the proteins synthesized. Thus, the proteins synthesized in the presence of threonine plus lysine may be hydrolytic enzymes involved in the acceleration of senescence. Excessive amounts of one amino acid may disrupt the editing mechanisms involved in tRNA aminoacylation by outcompeting the cognate amino acids at the active site of the synthetase (Fersht, 1981), or possibly by disrupting the co-ordination of amino acid and protein exchange between the chloroplast and cytoplasm.

Genetic studies have provided some evidence regarding the regulation of aspartate pathway in plants. Organs or tissues less sensitive to aspartate pathway end-products have been characterized in several reports, however only the work of

Hibberd *et al*. (1980) indicated an alteration in the regulatory properties (higher Ki for lysine) of lysine–sensitive aspartokinase from a lysine insensitive mutant isolated in tissue culture. This trait was inheritable in maize plants regenerated from the callus (Hibberd and Green, 1982). Aspartokinase from S (2–aminoethyl)–L–cysteine (a lysine analog) resistant carrot cell suspension cultures was only slightly less inhibited by lysine or threonine than the enzyme from normal cells (Matthews *et al*., 1980), suggesting another regulatory site. The possibility of genetic alteration of the tRNA or the synthetase in response to the analogue has not been considered. Future genetic and physiological studies may have to take into consideration the possible role of protein turnover and tRNA charging in the regulation of plant biosynthetic pathways. Unfortunately, nothing is known about the role amino acid availability and tRNA charging play in the regulation of protein turnover. This aspect of plant metabolism is examined in the following chapter.

4. Aminoacylation of tRNA and Protein Synthesis.

4.1 Introduction

Amino acid starvation in bacteria initiates the so called stringent response. The primary effect of the decreased amino acid availability is the decreased aminoacylation of tRNAs, resulting in a decreased rate of protein synthesis and increased rate of protein degradation (Yegian, *et al.*, 1966; Gallant, 1979; Cozzone, 1981). Bacteria of the genus *Bacillus* initiate sporulation in deficient media (Szulmajster, 1979). This developmental process also appears to be dependent on the stringent response (Lopez *et al.*, 1981).

A relationship between tRNA acylation and protein metabolism also exists in mammalian tissues, but seems to be more complex (Grummt and Grummt, 1976). Studies utilizing histidinol, a competitive inhibitor of his-tRNA synthetase, indicate that decreased his-tRNA charging results in decreased protein synthetic capacity, including polysome disaggregation, and increased protein degradation in cultured normal and mutant Chinese hamster ovary cells (Stanners *et al.*, 1978; Scornik *et al.*, 1980). In the same system, decreased aminoacylation of several tRNAs resulted in de-repression of asparagine synthetase activity, suggesting the existence of intermediary regulators (Andrulis *et al.*, 1979). Charging of rat liver tRNAs was not affected by fasting, only by feeding imbalanced, single amino acid-deficient diet (Allen *et al.*, 1969). However, at a 25% ile-tRNA charging level, protein synthesis decreased to only 66% of the control rate (Shenoy and Rogers, 1978). Formation of the 40-S initiation complex appears to be only indirectly regulated by uncharged tRNA (Austin, *et al.*, 1982). Moderate decreases in amino acid availability influenced the rate of translational elongation, while less than 50% charging was needed to affect initiation and polysome distribution in Ehrlich ascites cells (Ogilvie *et al.*, 1979). Apparently, protein synthesis and tRNA charging are protected from extreme substrate fluctuations in mammalian systems.

Charging of plant tRNAs with amino acids has not been investigated in relation to nutrient availability. Improved capacity for tRNA aminoacylation has been reported during breaking of dormancy by chilling of pear embryos (Tao and Khan, 1974) and in germinating lupin seed cotyledons (Kedzierski *et al.*, 1980). Changes in the tRNA complement were detected in senescing plant organs (Wright *et al.*, 1972/73; Pillay and

Cherry, 1974) and the ability of tRNAs to accept amino acids decreased with age of soybean cotyledons (Pillay and Gowda, 1981). The validity of this conclusion is difficult to assess since Tris buffer, which stimulates deacylation, was employed. Furthermore, the possibility of sub-optimal enzyme to tRNA ratios was not tested (Gusseck, 1974).

The consequences of decreased nutrient availability have been investigated in *Lemna minor* at the level of protein metabolism. Decreased rates of protein synthesis and increased rates of protein degradation were reported following transfer of actively growing plants into water or nitrate-, sulfate-, phosphate-, or magnesium-deficient media (Trewavas, 1972b; Cooke *et al.*, 1979). The following section attempts to establish the status of tRNA aminoacylation and of *in vivo* protein synthesis in growing versus senescing, nitrate- or sulfate-deficient plants.

4.2 Materials and Methods

4.2.1 Protein synthesis

Ten uCi of carrier free L-[4,5-^{3}H] leucine (Amersham, Illinois, 170 Ci/mmole) or L-[^{35}S] cysteine hydrochloride (Amersham, Illinois, 58 mCi/mmole) were added to 2 g of 10 day-old tissue in 10 ml of sterile medium of the same age. After 15, 30 or 60 minutes, excess radioactivity was removed by washing the plants 3x with 30 to 50 ml of water. Tissue was then homogenized and the protein extracted and estimated as described in Section 3.2.4.

4.2.2 tRNA charging with labeled exogenous leucine.

Following 30 minute feedings of tritiated leucine (as in Section 4.2.1), the tissue was homogenized in 15 ml of sodium acetate buffer (0.2 M, pH 5, containing 1 mM $MgCl_2$ and 1% w/v SDS) followed by addition of 20 ml of buffer-saturated phenol (Weil, 1979). The tubes were shaken vigorously for 60 minutes at room temperature. The two phases were separated by centrifugation at 200x g for 5 minutes. The upper phases were then collected. The remaining phenol layers were washed with 10 ml of the above buffer (without SDS) for an additional 30 minutes and the aqueous phase collected as above. The combined aqueous phases were re-extracted once more for 30 minutes with

20 ml of buffer saturated phenol. The aqueous fraction was treated with two volumes (50 ml) of 95% (v/v) ethanol and the nucleic acids precipitated at −20°C overnight and collected by centrifugation at 6,000x g for 10 minutes at 0°C. The pellet was dissolved in 5 ml of sodium acetate buffer (0.2 M, pH5, containing 10 mM $MgCl_2$) and treated for 30 minutes at room temperature with DNase (P−L Biochemicals, Wisconsin; RNase free, from bovine pancreas, 5 ug/ml final concentration). Remaining nucleic acids were precipitated with 10 ml of 95% ethanol at −20°C overnight. The 6,000x g pellet was dissolved in 10 ml of sodium acetate buffer (0.2 M, pH 5, 1 mM $MgCl_2$) and large molecular weight RNAs precipitated with 10 ml of 4 N NaCl (2 N final concentration) for 30 minutes. Transfer RNA remaining in the 6,000x g supernatant was precipitated as above. The resulting pellet was dissolved in 2 ml of 2 N TrisHCl buffer (pH 8.5) and heated to 37°C for 60 minutes to de−acylate the tRNA, precipitated overnight with 4 ml of cold ethanol and collected at 20,000x g for 10 minutes at 0°C (Andrulis and Arfin, 1979). Radioactivity of amino acids in the supernatant was determined as in Section 3.2.2 and the amount of tRNA (dissolved in water) estimated by absorbance at 260 nm (1 O.D.= 40 ug/ml). The purity of the extract was tested on 10% PAGE electrophoresis tube gel (Maxam and Gilbert, 1980; thanks are accorded to Mrs. D. McIntyre of the Microbiology Department for performing this analysis). Extracted plant tRNA co−migrated with authentic *E. coli* tRNA (Boehringer−Mannheim, Dorval). Some large nucleic acids remained at the origin and one unidentified band was present in the plant tRNA preparation.

4.2.3 Endogenous aminoacylation level of plant tRNA

The procedure for tRNA isolation was essentially as above, except 20 g of tissue were used and the reagent volumes were increased ten fold. Deacylation was achieved in 2N ammonium hydroxide at 50°C for 30 minutes and the excess ammonia was evaporated to dryness under vacuum (on a Buchler flash evaporator, Buchler Instruments, Fort Lee, New York) within further 10 to 20 minutes at 50°C. The residue was redissolved in 2 ml of sodium citrate buffer (0.2 N, pH 2.2) and precipitated overnight with 4 ml of 95% ethanol. Insoluble tRNA was collected by centrifugation at 20,000x g for 10 minutes and the supernatant was dried under vacuum and re−dissolved in 1 ml of the above citrate buffer for amino acid analysis (Section 2.2.3).

4.3 Results

With the exception of glycine, amino acid charging of tRNA was found to be slightly, but not significantly, decreased in the nitrate-deficient plants (Table 21). The large standard errors were due to analysis of amino acid concentrations at the detection limit of the amino acid analyzer's spectrophotometer. The trace amounts of methionine suggested charging levels comparable to those determined *in vivo* by an isotope dilution method in a related species, *Lemna minor* (Trewavas, 1972a) - 200 to 400 pmoles methionine per mg nucleic acid. The absolute amounts of tRNA per gram tissue were lower in the nitrate- and sulfate-deficient plants (Table 22). Within 14 hours of adding the lacking anion, tRNA levels remained statistically unchanged in both types of cultures recovering from deficiency.

Radiolabeled leucine was used to estimate the rate of tRNA charging *in vivo*. Increased uptake of label by deficient plants increased the labeling of tRNA, expressed on a fresh weight or tRNA content basis. However, the fraction of DPM in tRNA versus total taken up was less in the deficient plants (Table 22, see % of uptake). This may partially be due to decreased availability of tRNA in deficient plants. The increased labeling per mg tRNA from deficient plants may have been due to slow removal of label by protein synthesis. That this may be the case was indicated by the decreased specific labeling (to control level) by recovering plants, which took up amounts of label equivalent to the deficient plants (Table 22). Furthermore, as indicated in Table 23, relatively more label remained in the tRNA from deficient plants than from control plants following one hour "chase" in water. The opposite was the case in the recovering plants, in which the tritiated leucyl-tRNA label was lost more rapidly than in the controls (Table 23). This was in agreement with the rapid incorporation of labeled leucine into soluble protein of recovering plants (Fig. 4).

Protein content based on fresh weight was significantly lower in ten day-old deficient plants compared with controls of the same age, although after only 14 hours of recovery, protein content of the deficient plants increased significantly (Table 24). The labeling in the 20,000g pellet protein was comparable to that in the soluble protein and will not be considered further.

Table 21. *The level of tRNA charging by endogenous amino acids in 10 day-old control and nitrate-deficient plants.*

	Deficiency	
	None	Nitrate
	nmoles x mg tRNA^{-1}	
Aspartate	5.24 ± 1.64[1]	5.21 ± 0.99
Threonine	0.74 ± 0.24	0.46 ± 0.21
Serine[2]	3.05 ± 0.88	2.57 ± 0.68
Glutamate	1.51 ± 0.49	0.82 ± 0.15
Glycine	3.94 ± 1.16	4.25 ± 1.23
Alanine	1.27 ± 0.42	0.94 ± 0.24
Valine	0.71 ± 0.27	0.32 ± 0.11
Methionine	trace	trace
Isoleucine	0.48 ± 0.18	0.27 ± 0.08
Leucine	0.48 ± 0.20	0.29 ± 0.10
Tyrosine	trace	trace
Phenylalanine	trace	trace
Total	17.42 ± 5.25	15.10 ± 2.05

Procedures are described in Materials and Methods.

[1]Each number represents the average of five extracts ± S.E.M.

[2]Includes glutamine and asparagine.

Table 22. *L-[4,5 - ^{3}H] leucine charging of tRNA from 10 day old control, deficient and recovering plants.*

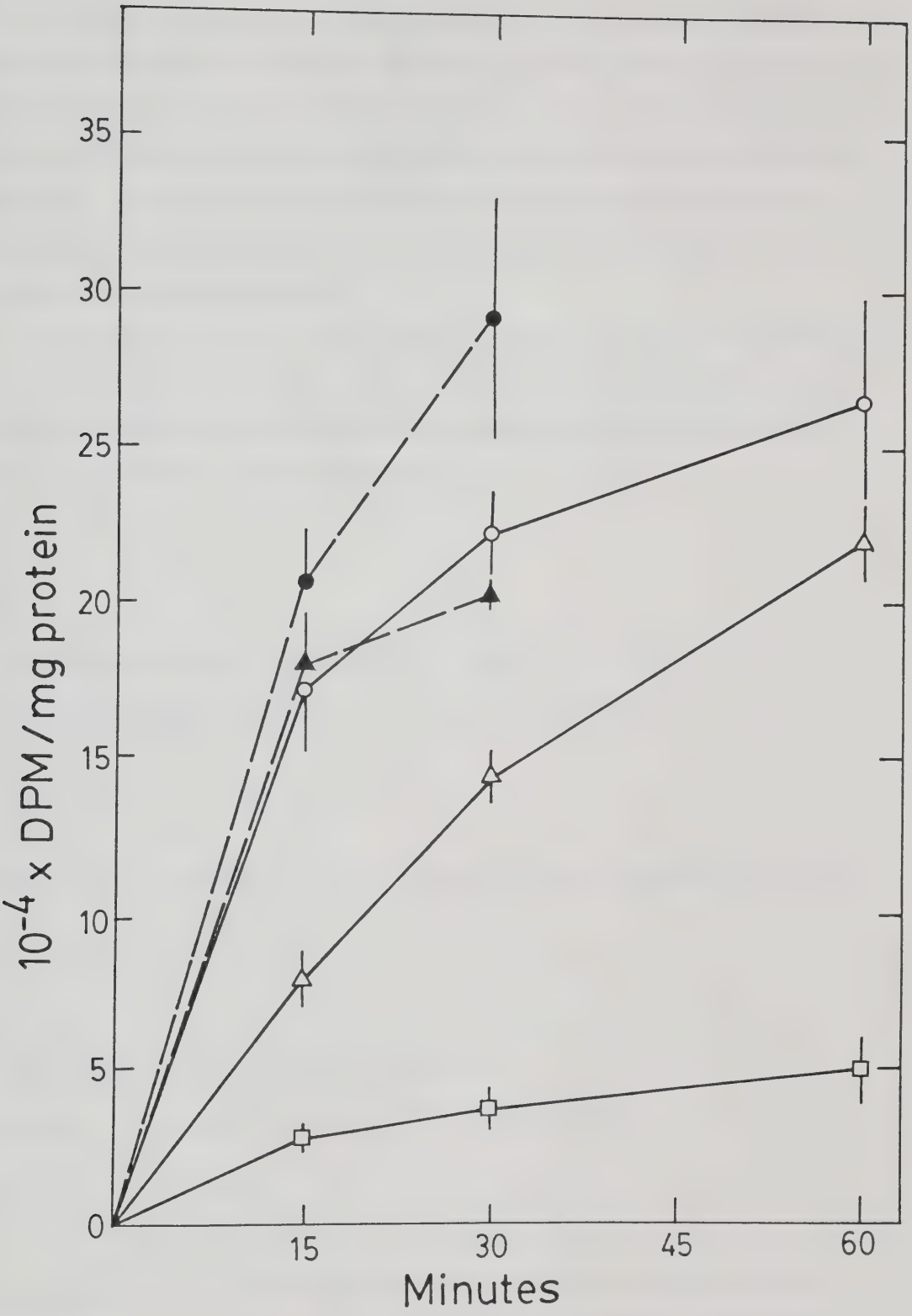
	tRNA content	tRNA charging at 30 minutes		tRNA charging as % of uptake	Uptake at 30 minutes
	μg tRNA x g f.wt.$^{-1}$	*DPM x mg tRNA^{-1}*	*DPM in tRNA x g f.wt.$^{-1}$*	%	*Total DPM x g f.wt.$^{-1}$*
Deficiency					
None	511 ± 44[1]	8,287 ± 1,205	3,968 ± 214	0.15	2,505,700 ± 228,300
Nitrate	310 ± 58	19,850 ± 3,305	5,468 ± 190	0.10	5,461,200 ± 537,200
Nitrate-recovering[2]	312 ± 16	10,393 ± 794	3,214 ± 188	0.06	5,424,300 ± 77,000
Sulfate	240 ± 15	19,170 ± 5,885	4,485 ± 1,560	0.09	4,762,800 ± 535,800
Sulfate-recovering[2]	284 ± 16	8,736 ± 4,125	2,445 ± 947	0.05	4,505,300 ± 612,100

Procedures are described in Materials and Methods

[1]Each number represents an average of at least four extracts ± S.E.M.

[2]Nitrate-deficient cultures were aseptically given 5 ml of 40 mM $Ca(NO_3)_2$ and KNO_3 (final 5 mM each) and sulfate-deficient cultures were provided with 5 ml of 16 mM $MgSO_4$ (final 2 mM) for 14 hours.

Figure 4. *Incorporation of ^{3}H-leucine into cold TCA precipitated protein. For procedures see Materials and Methods. Control (□), nitrate-deficient (0)and sulfate-deficient (△); solid symbols (●,▲) - plants recovering in the prescence of nitrate or sulfate, respectively. Each point is an average of two extracts, bars represent S.E.M.*


35
30
25
20
15
10
5
0
15
30
45
60
Minutes
10^{-4} x DPM/mg protein

As was the case for tRNA labeling, the incorporation of tritiated leucine into soluble protein appeared to be higher in the deficient plants than the controls (Fig. 4). This was accompanied by increased uptake by the deficient cultures. Relating incorporation to uptake (Fig. 5), it became clear that in the deficient plants, protein synthesis was either comparable to, or less than, occured in the controls. Recovering plants incorporated more label than the controls (Fig. 5). The non-linear kinetics of incorporation into protein were due to rapid (parabolic) uptake.

To compare the capacity of non-deficient, nitrate-deficient and recovering plants further, a method for evaluating the rates of protein synthesis and degradation proposed by Trewavas (1972a), and Reiner (1953) was employed. To use the nomenclature of Trewavas, this relationship holds during labeling:

$$\frac{dS_p}{dt} = (S_t - S_p) \times \frac{V_s}{P}$$

where:

S_p = specific activity in protein, S_t = specific activity in tRNA,

V_s = rate of protein synthesis, P = amount of protein, t = time,

$\frac{V_s}{P} = K_s$ or rate constant of synthesis.

The rate of protein accumulation in the culture is determined experimentally and since:

$$\frac{dP}{dt} = (K_s - K_d) \times P$$

where:

K_d = rate constant of protein degradation

the term $K_s - K_d$ can be determined from the slope of:

$$\log P = (K_s - K_d) \times t + C$$

Assumptions necessary for the application of this expression are that synthesis and degradation of protein are (pseudo) first order reactions and that the system is in a steady state. The latter does not hold for the recovering culture and Ks is only given as a

Table 23. *Rate of L-[3,4 - ^3H] leucine turnover in the leucyl tRNA pool.*

	tRNA charging at 90 minutes[1]	Charging at 90 minutes as % charging at 30 minutes (From Table 22)
	DPM x mg tRNA^{-1}	%
Deficiency		
None	2,549 ± 130[2]	30
Nitrate	10,318 ± 1,314	52
Nitrate-recovering[3]	2,300 ± 68	22
Sulfate	7,200 ± 198	38
Sulfate-recovering[3]	2,330 ± 43	27

Procedures are described in Materials and Methods.

[1]After 30 minutes of feeding, samples were washed off 3 x with 30 - 50 ml of sterile distilled water, further 10 ml were added and the samples were kept for additional 60 minutes. tRNA was extracted following this "chase" period.

[2]Each number represents the average of two extracts ± S.E.M.

[3]Nitrate-deficient cultures were aseptically given 5 ml of 40 mM $Ca(NO_3)_2$ and KNO_3 (final 5 mM each) and sulfate-deficient cultures were provided with 5 ml of 16 mM $MgSO_4$ (final 2 mM) for 14 hours.

Table 24. *Protein content of 10 day old control, deficient and recovering plants.*

Deficiency	Protein
	mg · g fwt. $^{-1}$
None	14.30 ± 0.50[1]
Nitrate	5.38 ± 0.28
Nitrate-recovering[2]	6.01 ± 0.26
Sulfate	5.75 ± 0.31
Sulfate-recovering[2]	7.30 ± 0.24

Procedures are described in Materials and Methods.

[1]Each number is an average of at least six extracts ± S.E.M.

[2]As in Table 2, after 14 hours.

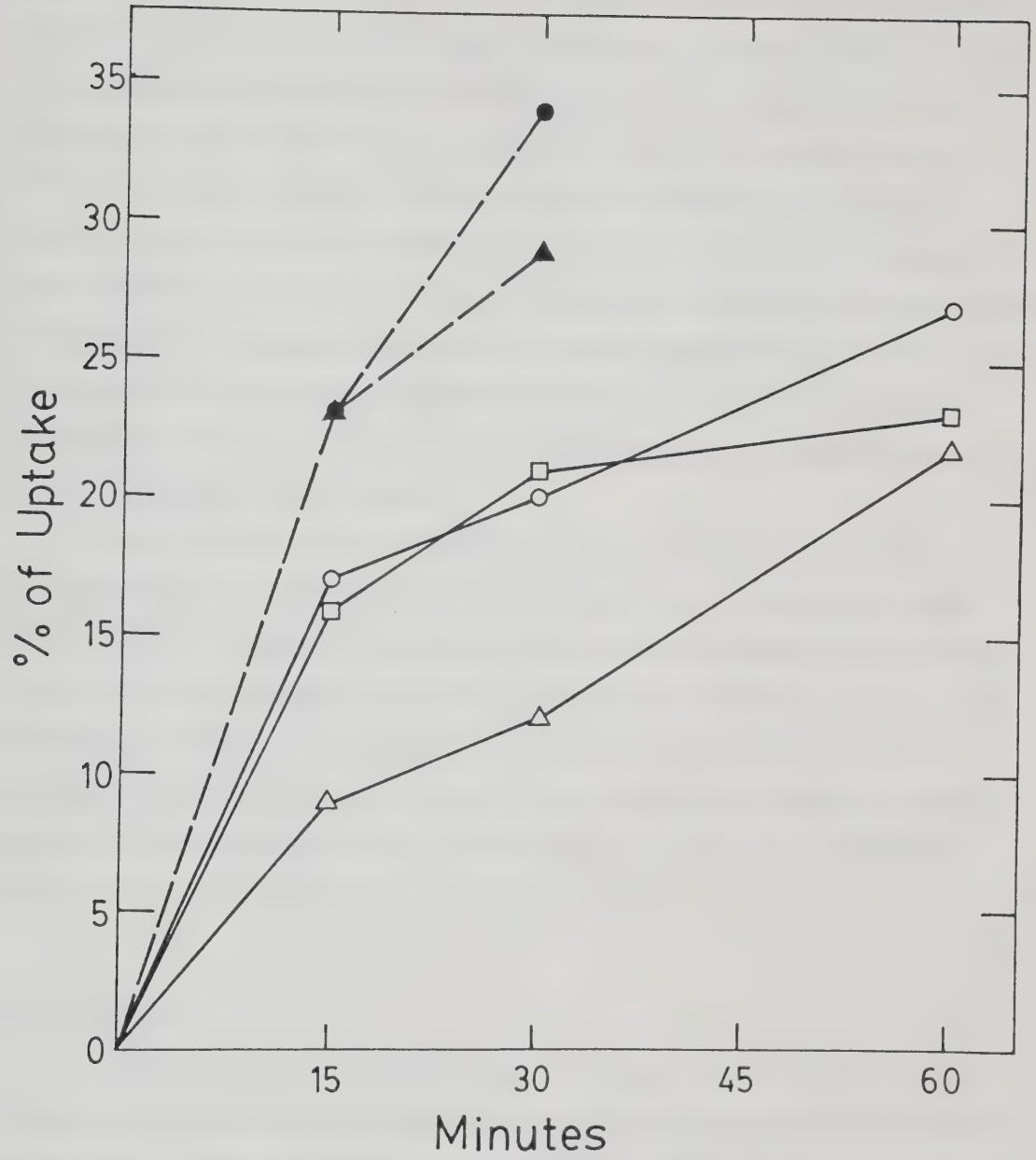


Figure 5. *Incorporation of ^{3}H-leucine into cold TCA precipitated protein. Data are expressed as a percent of total uptake (DPM/g f. wt. at each point of time). For procedures, see Materials and Methods. Legend as in Figure 4.*

range of possible values. Furthermore, the slopes of logP vs. time were only approximations, since insufficient data were collected. Additional limitations of this method are the non-uniform rates of frond senescence and the possible differential loss of protein synthetic capacity within cellular compartments. The rate constants represent only average values characteristic of the culture as a whole. The approximate rate constants calculated in Table 25 are presented for comparative purposes, since they correlate the specific labeling of tRNA and protein and deal with the problem of dissimilar precursor uptake. The synthetic rate constants indicate that the capacity of nitrate-deficient plants to synthesize protein was less than that of the controls, while the recovering plants exceeded the synthetic rates of the controls. The rate of degradation of protein was several fold greater in the deficient plants than in the controls. Small differences between Ks and Kd reflected the lack of protein accumulation between day 7 and 11 in the nitrate-deficient plants.

The incorporation of ^{35}S-cysteine into soluble protein was also investigated in view of the negative effects of exogenous cysteine on growth (Table 3), decreased cysteine content in protein of deficient plants (Table 8) and the importance of cysteine in chloroplastic protein (Hanson *et al.*, 1941). Incorporation of labeled cysteine was also non-linear over 60 minutes, possibly due to rapid catabolism, in addition to rapid, non-linear uptake (Figure 6). Incorporation relative to uptake was limited in the deficient plants (Fig. 7) and comparable to the control in the recovering plants. In the latter, the specific incorporation was less than in the controls (Fig. 6).

4.4 Discussion

The ability of nutrient-deprived bacteria to regulate the slow-down of their metabolism to "maintenance levels" by the relative amino acid charging of tRNA (Gallant, 1979; Cozzone, 1981) poses the possibility that similar regulatory mechanisms may exist in eukaryotic organisms, including plants. In the absence of plant mutants, deficient in the aminoacylation reactions, one has to rely on evidence obtained with nutrient deficient organisms or cells.

Dramatic decrease in tRNA charging, to 25% from the usual 75 to 90%, can be expected in amino acid starving *E. coli* (Yegian *et al.*, 1966). Clearly, changes of this

Table 25. *Calculation of rate constants for protein synthesis (K_s) and degradation (K_d) in control, nitrate-deficient and recovering plants.*[1]

	Deficiency		
	None	Nitrate	Nitrate-recovering[2]
Change in protein specific activity $\frac{dSp}{dt}$ at 30 minutes			
DPM x hr^{-1} $pmole^{-1}$	0.06	0.20	0.25
Specific activity in leu tRNA			
DPM x $pmole^{-1}$	17	68	35
Rate constant of synthesis (K_s)[3]			
hr^{-1}	0.0035	0.0029	0.007 to 0.010
day^{-1}	0.084	0.070	0.168 to 0.240
Slope of log P vs. time ($K_s - K_d$)			
day^{-1}	0.075	0.003	
Rate constant of degradation (K_d)			
day^{-1}	0.009	0.067	

[1]Determined according to Trewavas (1972a), at 30 minutes of leucine feeding.

[2] Nitrate-deficient cultures were aseptically given 5 ml of 40 mM $Ca(NO_3)_2$ and KNO_3 (final 5 mM each) and sulfate-deficient cultures were provided with 5 ml of 16 mM $MgSO_4$ (final 2 mM) for 14 hours.

[3] Determined from $\frac{dSp}{dt} = S_t \times K_s$, since Sp (specific activity in protein) was negligible.

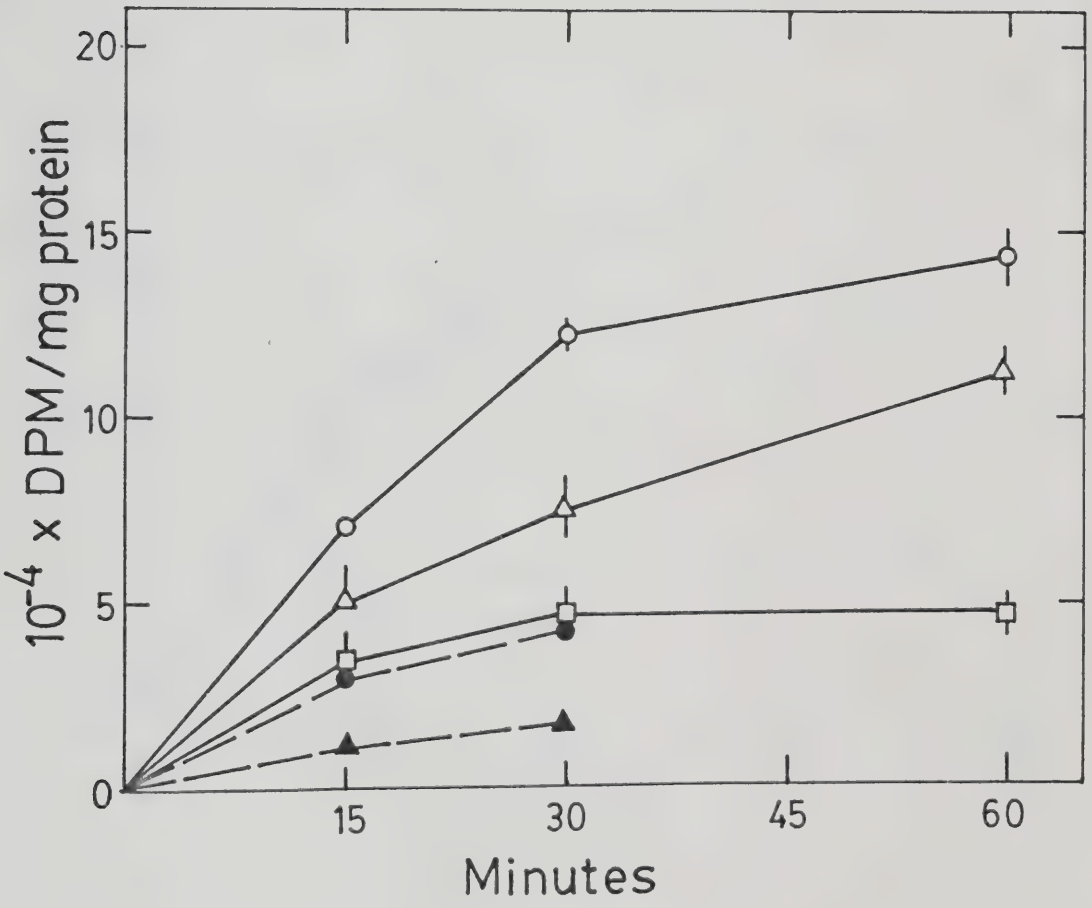


Figure 6. *Incorporation of ^{35}S-cysteine into cold TCA precipitate protein. For procedures see Materials and Methods. Legend as in Figure 4. Where not shown, S.E.M. did not exceed the size of the symbol.*

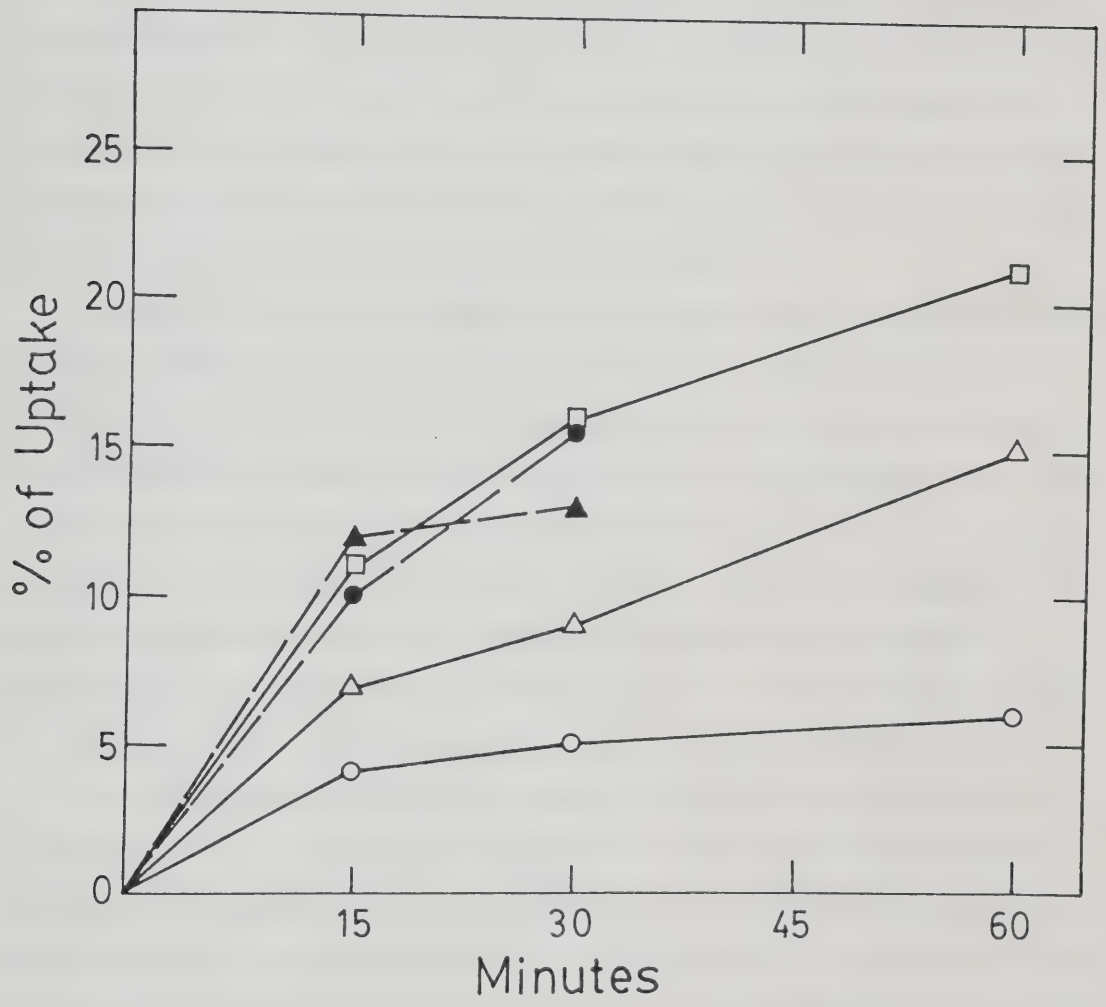


Figure 7. *Incorporation of ^{35}S-cysteine into cold TCA precipitate, expressed as percent of total uptake (DPM/g f. wt. at each point of time). For procedures see Materials and Methods. Legend as in Figure 4.*

magnitude were not observed in nitrate-deficient *Spirodela*, in which average charging decreased by only 13%, a change which was statistically not significant (Table 21). The decreased charging by exogenous tritiated leucine may have resulted from decreased availability of tRNA rather than the rate of charging (Table 22). Furthermore, the lower turnover of label in the tRNA pool of the deficient *Spirodela* (Table 23) may be either the cause, or the result, of the slightly decreased rate of protein synthesis (Table 25), although the latter is not likely in view of the dependence of protein synthesis on available substrate – aminoacylated tRNA. The response of plants recovering from nitrate deficiency indicates that despite lower tRNA levels, these plants were capable of increasing the rate of amino acid flux through tRNA (Tables 22 and 23) and the rate of protein synthesis (Table 25). Thus, substrate availability may be the main factor limiting protein synthesis in nitrate- and sulfate-deficient plants.

The role of tRNA in plants appears to be as complex as in animal tissues. Firstly, liver tRNA charging during fasting (amino acid starvation) was not decreased (Allen *et al.*, 1969), secondly, inhibition of the initiation and elongation steps of protein synthesis required substantial decreases in tRNA charging (Ogilvie *et al.*, 1979; Austin *et al.*, 1982) and thirdly, protein degradation was greatly stimulated by decreased tRNA charging levels (Stanners *et al.*, 1978; Scornik *et al.*, 1980; Austin *et al.*, 1982). It was suggested by the above studies that decreased tRNA charging in muscle tissue and the resulting degradation of muscle protein may provide amino acids for the synthesis of proteins in other tissues, in which tRNA aminoacylation must be "buffered" from fluctuations.

In *Spirodela*, nutrient deficiency appeared to have more effect on protein degradation than on protein synthesis (Table 25). However, the possible relationship between tRNA charging and protein degradation remains to be resolved. In particular, the question remains: which proteins can a plant sacrifice without compromising its ability to recover from the deficiency? A secondary function of chloroplastic proteins, particularly RuBP carboxylase/oxygenase, appears to be that of a leaf storage protein, which is readily mobilised during senescence (Eilam *et al.*, 1971; Callow *et al.*, 1972; Peterson *et al.*, 1973; Callow, 1974; Peterson and Huffaker, 1975; Peoples *et al.*, 1980; Morita, 1980; Miller and Huffaker, 1982). It seems reasonable to assume, that the same protein(s) is(are) degraded in response to nutrient deficiency, particularly since the chloroplast is

the first organelle to deteriorate structurally during senescence (Butler and Simon, 1970) and during nutrient deficiency (Vesk *et al.*, 1966). The function of the chloroplast in primary nitrate and sulfate assimilation may cause it to be more sensitive to deficiency of these anions. Even though the chloroplasts contain about 50% of the cellular protein, only a small fraction of this protein is actually synthesized in the chloroplast, (Cifferi, 1978). Thus the primary decrease in glutamate or cysteine availability may initiate regulatory events similar to the stringent response of bacteria, and this possibly significant decrease in chloroplastic tRNA charging and protein synthesis would not be detectable due to the "dilution effect" by active cytoplasmic and/or mitochondrial protein synthesis. The relative contribution of organelles to overall cellular protein synthesis at different stages of development remains to be evaluated.

Two additional points deserve mention. Firstly, the different rate constants of protein accumulation obtained in this study 0.075 day^{-1}, in *Lemna minor* 0.39 day^{-1} (Trewavas, 1972b), 0.33 day^{-1} (Cooke *et al.*, 1979) and in tobacco callus, 0.17 day^{-1} (Kemp and Sutton, 1971) may reflect the actual potential for growth of these tissues under the culture conditions used by the respective investigators (for example the presence of sugars in the medium). Secondly, although the rate constants of protein synthesis and degradation were not determined for the sulfate-deficient plants, uptake and incorporation into the TCA insoluble fraction followed similar patterns to those observed for nitrate-deficient plants. For example, the apparent rates of incorporation into protein on day 10 indicated that sulfate-deficient plants were not as severely affected as the nitrate-deficient plants. The recovering plants utilized labeled leucine and cysteine to differing extents. Cysteine may be synthesized *de novo* more rapidly than leucine in the recovering plants, diluting the exogenous labeled cysteine more than the exogenous leucine would be diluted by endogenous leucine. Another possibility is that cysteine is used preferentially in the synthesis of S-rich chloroplastic protein(s), which may be expected to exceed the cytoplasmic protein synthesis during recovery.

In conclusion, it was demonstrated that tRNA charging decreased during nitrate-deficiency and increased during recovery from this stress. As expected, the aminoacylation reaction represents a link between amino acid availability and protein turnover, although the possible regulatory role of tRNA remains to be established. Protein

degradation, rather than synthesis, was found to be affected by decreased nitrate availability.

5. General Conclusions

Although conclusions reached in this study were already stated in the foregoing chapters, the purpose of this section is firstly to summarize these conclusions in the general context of macro-nutrient deficiency and secondly to identify and discuss some areas for future research.

1) Nitrate and sulfate deficiencies initiated frond senescence (i.e. chlorophyll and protein degradation) and turion development in *Spirodela*. Applications of the lacking anions, or amino acids that are known to be products of assimilation, reversed these developmental processes and supported vegetative growth.

2) Turions which formed in nitrate- or sulfate-deficient media were dormant, i.e. did not germinate in water or nutrient media under otherwise favorable environmental conditions.

3) Osmotic treatment in 20%(w/w) PEG 6000 broke the turion dormancy, only to permit germination in the anion which was lacking during turion development or in an amino acid which would be synthesized from that anion, but not in water.

4) Cytokinins appeared to break turion dormancy completely, stimulating germination in the absence of exogenous nutrients.

5) Exogenous cytokinins also reversed turion development in nutrient-deficient media and stimulated vegetative frond development, although senescence, measured by chlorophyll content, was not reversed.

6) The overall concentration of protein declined in both, nitrate- and sulfate-deficient plants, although total protein content per culture remained nearly constant between 7 to 11 days of culture, suggesting that re-distribution of protein-bound amino acids from older fronds into meristems was taking place. The approximate rate constant of proteolysis was calculated to be 0.009 day^{-1} in the controls, compared to 0.067 day^{-1} in the nitrate-deficient plants.

7) The concentrations of detectable free amino acids were much lower in the nitrate-deficient plants than in controls. Sulfate-deficient plants maintained levels of free amino acids comparable to the controls, with the exception of markedly increased levels of asparagine and glutamine.

8) Sulfate-deficient plants did not contain significantly less free methionine than control or nitrate-deficient plants.

9) Activity of the aspartate pathway, measured by *in vivo* conversion of labeled aspartate, homoserine and cysteine into threonine, isoleucine and methionine did not appear to be significantly impaired by deficiency of either anion, even though the assimilatory pathways of both anions are linked at the step of homocysteine synthesis (Figure 3).

10) *In vitro* activity of homoserine dehydrogenase extracted from deficient plants was about 10 fold lower than that from control plants, yet it was sufficient to maintain synthesis in the deficient plants.

11) Exogenous threonine plus lysine inhibited methionine synthesis and also affected protein synthesis. Exogenous glutamate, aspartate and glycine supplied to control plants enhanced turion development, apparently interfering with normal metabolism.

12) Aminoacylation level of total cellular tRNA from nitrate-deficient plants was 13% less than in the controls, statistically not significant change.

13) The rate constant of overall cellular protein synthesis was only 12% lower in the nitrate-deficient plants than in the controls, while the rate constant of protein synthesis in plants recovering from nitrate deficiency for 14 hours was 100% higher than that in the controls. Protein synthesis of sulfate-deficient plants did not appear to be significantly impaired.

14) Protein and tRNA concentrations were significantly lower in both types of deficient plants than in the controls after 10 to 11 days in culture. A statistically significant increase in protein content was detected only after 14 hours of recovery in the appropriate anion. The tRNA level increased significantly after 14 hours in recovering sulfate-deficient plants, but not in recovering nitrate-deficient plants.

15) In comparison with the controls, the turnover of ^{3}H-leucine in the tRNA pool was slower in the deficient plants and more rapid in the recovering plants.

It was proposed in the General Introduction, that macro-nutrient deficiency, or the inability to utilize nutrients, may be the underlying cause of senescence under normal

environmental conditions. The two molecular events characteristic of foliar senescence, i.e. proteolysis and chlorophyll degradation, were also observed in leaves senescing in response to both, nitrate and sulfate deficiencies. Consequently, elucidation of the metabolic and regulatory events occurring in deficient plants may provide knowledge applicable to studies of senescence under normal environmental conditions.

The *Spirodela* system offers opportunities for innumerable studies of nutrient deficiencies, intermediary metabolism and macromolecular metabolism. However, in the following paragraphs, only those experiments will be suggested, which seem to be a logical continuation of experiments reported in this study. The initial experiments dealing with developmental consequences of nitrate and sulfate deficiency and the effects of anion, amino acid or cytokinin supplements have defined a broad range of events which require characterisation at the organellar and molecular levels.

Several lines of evidence suggest that chloroplast function is an early target (or cause) of foliar senescence. Chloroplast structure and numbers are affected early in senescence (Butler and Simon, 1970) or nutrient deficiency (Vesk *et al.*, 1966). Chlorophyll loss is one of the clearest measures of foliar senescence (Thimann, 1980) and chloroplastic proteins, particularly RuBP carboxylase/oxygenase, are the major source of free amino acids in senescing leaves (Morita, 1980; Peoples *et al.*, 1980). Furthermore both, the assimilation of nitrate into glutamine and sulfate into cysteine are chloroplastic events (Miflin and Lea, 1976; Giovanelli *et al.*, 1980), suggesting that deficiencies of these anions may initially affect metabolism of the chloroplast. For these reasons, it may be most rewarding to focus future investigations on the chloroplast, although the close relationship between cytoplasmic and chloroplastic metabolism should be kept in mind.

A detailed electron-microscopic study of the key developmental stages in senescing and recovering chloroplasts may be a pre-requisite necessary for subsequent biochemical studies. Following this, the problem of primary interest will be a direct demonstration of decreased capacity for *de novo* synthesis of glutamate and cysteine by chloroplasts in early stages of deficiency. Such a study may be difficult, since applications of labeled precursors effectively reverse the deficient condition. Perhaps studies using reciprocal feedings, i.e. labeled sulfate to nitrate-deficient plants or labeled

nitrate to sulfate-deficient plants will provide some answers, in view of the close link between the pathways at the level of cysteine synthesis. Difficulties with uptake of the isotopic species into already saturated pools may be encountered. Furthermore, the use of inhibitors of the assimilatory steps (for example methionine sulfoximine) may help in further elucidation of the role of assimilatory pathways in senescence.

Macromolecular metabolism in deficient *Spirodela* may be investigated even in the absence of evidence for limited amino acid availability. Electrophoresis and isoelectric focusing methods could be employed to identify chloroplastic proteins involved in senescence, by virtue of their stability or instability. Such proteins, or other large or easily separated and well characterised proteins may be used in subsequent detailed investigations of turnover, using methodology employed for specific animal proteins such as ovalbumin (Palmiter, 1975) or myosin (Martin *et al.*, 1977). These methods rely on the knowledge of the rates of turnover of mRNA, tRNA, amino acids in the tRNA pool and the concentration of protein in question which can then be used to calculate the absolute rates of synthesis and degradation of this protein. Charging levels of chloroplastic tRNA could be measured using the periodate oxidation of non-acylated tRNA (Yegian *et al.*, 1966; Andrulis and Arfin, 1979) concentrating for simplicity on key tRNAs and corresponding synthetases. This approach would give some indication of not only the rates of specific protein turnover, but also the possible role of tRNA aminoacylation and tRNA availability in its regulation.

The possibility that the primary effects of decreased amino acid synthesis by the chloroplast take place in the cytoplasm cannot be excluded, especially in view of the observation that many chloroplastic proteins are synthesized in this compartment, including the chloroplastic aminoacyl-tRNA synthetases (Cifferi, 1978). It may be difficult to analyse the total cytoplasmic protein complement, and stress could be placed on chloroplastic proteins originating in the cytoplasm. These can be identified using inhibitors of protein synthesis specific for 70S or 80S ribosomes, or in the light driven, *in vitro* chloroplastic protein synthesizing system (Blair and Ellis, 1973; Eaglesham and Ellis, 1974).

Cytokinin effects on *Spirodela* may be also speculatively linked to chloroplast function, in view of accumulating evidence for cytokinin involvement in chloroplast

development (Feierabend, 1981; Parthier *et al.*, 1981; Peaud-Lenoel and Axelos, 1981). A better understanding of tRNA function in plant protein synthesis may facilitate studies on the link between the function of tRNA and the occurrence of a cytokinin moiety on the 3' side of the anticodon of tRNAs responding to codons starting with uridine, i.e. the termination codons UAA, UAG, UGA and those coding for leu, cys, ser and aromatic amino acids (Skoog and Schmitz, 1979). Although the cytokinin moiety appears to be ubiquitous to tRNAs of many organisms, green plants may have evolved a system capable of modulating activity of these tRNAs (via free cytokinins?) in relation to plastid development. Unfortunately, the only study which demonstrates a functional role of the cytokinin moiety in tRNA comes from a triple mutant of yeast (Laten *et al.*, 1978). In this organism, the first mutation generates the UAA termination codon, the second mutation suppresses the termination codon by insertion of tyrosine carried by an isopentenyl adenine containing tRNA which responds to the UAA codon and the third mutation lowers the isopentenyl content of all tRNAs to 1.5% of wild type, resulting in reduced suppressor activity. In other words, the presence of isopentenyl adenine in the anti-codon region of the suppressor tRNA was needed to de-code the UAA codon as specifying tyrosine. It is possible that in plants, under certain circumstances, tRNAs containing cytokinins insert some of the above amino acids into the sites specified by termination codons, allowing synthesis of larger, functional proteins. Free cytokinins may compete with such tRNAs for binding sites on the ribosome. It should be stressed here, that decreased availability of charged tRNA in deficiency will have exactly opposite effect on codon usage, i.e. codons normally translated as specifying an amino acid will be read as translation termination codons. Initially, analysis of the quality of translation products from deficient or cytokinin treated plants may be possible using RuBP carboxylase/oxygenase as a model, although only some "senescence specific" proteins may be regulated in this manner.

Inhibitory effects of exogenously applied amino acids on growth observed in this study and by others (Fukunaga and King, 1982) can be also investigated further using *Spirodela*. It is likely that excessive availability of one or a few amino acids will increase the frequency of errors in tRNA charging (Fersht, 1981), resulting in the synthesis of faulty proteins. Analyses of tRNA charging and the rate and quality of proteins made

under these conditions are needed.

In conclusion, the withdrawal of macro-nutrients, or conversely, the application of exogenous amino acids to cultures of *Spirodela*, represent methods which effectively disrupt metabolism at a level closely linked to macromolecular synthesis. As such, these methods may serve as useful tools in elucidating the metabolic and regulatory mechanisms underlying plant development. Nutrient deficiency investigations in *Spirodela* can be expanded to include studies of plant growth regulators and other developmental phenomena such as turion dormancy and turion germination, in addition to frond senescence.

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